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The endocannabinoid system in female reproduction: characterization of major endocannabinoid-binding receptors expression and function during mouse oocyte meiotic maturation

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Summary

Introduction

Endocannabinoid system (ECS) includes lipid messengers termed endocannabinoids (eCBs), their receptor (CBRs) targets type-1 (CB_1) and type-2 (CB_2) cannabinoid receptors, G-protein coupled receptor 55 (GPR55), transient receptor potential vanilloid type 1 channel (TRPV1) and a number of metabolic enzymes. ECS has a key-role in virtually all steps of female reproduction. To date, among the 4 main receptors, only 2 receptors have been extensively studied in the mammalian oocytes, i.e. CB_1 and CB_2, both modulated during meiotic maturation. The aim of this thesis was (a) to determine expression levels of all these CBRs and (b) to investigate their role in the process of mouse oocyte meiotic maturation.

Methods

Adult CD1 female mice were primed with 5 IU PMSG and sacrificed: (a) 44 h later to obtain GV oocytes, (b) 8 or 12h after hCG (5 IU) injection to obtain MI or MII oocytes. CBRs mRNA and protein contents were assessed by qRT-PCR and Western blot; CBRs localization by confocal microscopy. CB_1, CB_2 and GPR55 roles during meiotic resumption (germinal vesicle breakdown, GVBD) or maturation up to MI and MII stages were assessed by incubating oocytes in the presence/absence of receptor antagonists (SR1, SR2, and ML193, respectively). cAMP concentration was assessed by EIA kit; MI/MII spindle morphology by appropriate immunofluorescent antibodies. Experiments were repeated at least 3 times.

Results

Despite a significant decrease of CB_1, CB_2 and GPR55 mRNAs occurring after GVBD, CB_2 and GPR55 protein contents increased significantly from GV to MI and MII. At GV, only CB_1 was localized in the oolemma, although it disappeared at MI. TRPV1 (mRNA/protein) was always undetectable. When oocytes were in vitro matured with CB_1 and CB_2 antagonists, a significant delay of GVBD was recorded, sustained by higher intraoocyte cAMP concentration. Although CBRs antagonists did not affect polar body I emission nor chromosome alignment at metaphase I or II plates, ML193 impaired the formation of normal MI or MII spindles in about 70% of oocytes. Indeed, ML193-

incubated oocytes showed a significant reduction of spindle length as compared with control.

Conclusion

In mouse oocytes, all the major eCB-receptors are differentially expressed during meiotic maturation. CB_1 and CB_2 have a prominent role in the control of meiosis resumption, while GPR55 could be involved in the assembly of MI and MII spindles. These findings offer potentially novel biomarkers involved in the physiological maturation of mouse oocytes and could be a starting point for investigating female fertility issues also in women.

Sommario

Introduzione

Il sistema endocannabinoide (ECS) include messaggeri lipidici chiamati endocannabinoidi (eCBs), i loro recettori (CBR) cannabici di tipo 1 (CB_1) e 2 (CB_2), il recettore accoppiato a proteine G 55 (GPR55), il transient receptor potential vanilloid type 1 channel (TRPV1) e gli enzimi metabolici. L'ECS ha un ruolo chiave in tutti i processi della riproduzione femminile. Ad oggi, tra i 4 principali recettori, solo 2 sono stati estensivamente studiati negli ovociti di mammifero, i.e. CB_1 e CB_2, entrambi modulati durante la maturazione meiotica. Lo scopo di questa tesi è stato quello di (a) determinare i livelli di espressione di questi CBR e (b) di identificare il loro ruolo nel processo di maturazione meiotica in ovociti di topo.

Metodi

Femmine di topo CD1 adulte sono state iniettate con 5 IU di PMSG e sacrificate: (a) 44 h dopo per ottenere ovociti allo stadio di GV, (b) 8 or 12 h dopo iniezione di hCG (5 IU) per ottenere ovociti allo stadio di MI o MII. I contenuti di mRNA e proteine dei CBRs sono stati valutati tramite qRT-PCR e Western blot; la loro localizzazione mediante microscopia confocale. Il ruolo di CB_1, CB_2 e GPR55 durante la ripresa meiotica (rottura della vescicola germinale, GVBD) o i processi di maturazione fino allo stadio di MI e MII sono stati valutati mediante l'incubazione degli ovociti in presenza di antagonisti specifici dei recettori (SR1, SR2 and ML193, rispettivamente). La concentrazione di cAMP è stata valutata mediante un kit EIA; la morfologia dei fusi di ovociti allo stadio di MI/MII è stata valutata mediante l'uso di idonei anticorpi immunofluorescenti. Gli esperimenti sono stati ripetuti almeno 3 volte.

Risultati

Nonostante una diminuzione significative degli mRNA dei recettori CB_1, CB_2 e GPR55 dopo la GVBD, il contenuto proteico di CB_2 e GPR55 aumentava significativamente da GV a MI e MII. Allo stadio di GV, solo CB_1 è stato localizzato sull'oolemma, mentre scompariva allo stadio di MI. TRPV1 (mRNA o proteina) non è mai stato rilevato. Quando gli ovociti sono stati maturati *in vitro* in presenza degli antagonisti di CB_1 e CB_2 è stato rilevato un rallentamento nella GVBD, sostenuto da maggiori concentrazioni di

cAMP intraovocitario. Sebbene gli antagonisti dei CBR non avessero effetti sull'estrusione del globulo polare o sull'allineamento dei cromosomi sulla piastra metafasica in MI o MII, ML193 comprometteva la formazione di fusi meiotici normali a MI o MII in circa il 70% degli ovociti. Infatti, gli ovociti incubati con ML193 mostravano una riduzione significativa della lunghezza del fuso rispetto al controllo.

Conclusioni

Negli ovociti di topo i principali recettori di eCB sono espressi diversamente durante la maturazione meiotica. I recettori CB_1 e CB_2 hanno un ruolo prominente nel controllo della ripresa meiotica, mentre GPR55 sembra essere coinvolto nell'assemblamento del fuso di ovociti allo stadio di MI e MII. Questi dati offrono potenziali nuovi biomarcatori coinvolti nella fisiologia della maturazione meiotica di ovociti di topo e potrebbero offrire spunti iniziali per lo studio di problematiche della fertilità femminile anche nelle donne

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List of abbreviations

17β-HSD: 17β-hydroxysteroid dehydrogenase

2-AG: 2-arachidonoylglycerol

3β-HSD: 3β-hydroxysteroid dehydrogenase

ABHD: α/β-hydrolase domain protein

AC: adenylyl cyclase

AEA: N-arachidonoylethanolamine, anandamide

AFs: antral follicles

Akt: protein kinase B

AMH: anti-Müllerian hormone

AMT: anandamide membrane transporter

AR: androgen receptors

AREG: amphiregulin

ART: assisted reproductive technologies

AURKA: Aurora-A protein kinase

AURKB: Aurora-B protein kinase

AURKC: Aurora-C protein kinase

BDNF: brain-derived neurotrophic factor

BF: blastocoelic fluid

BIRC5: baculoviral IAP repeat-containing protein 5

BMP-15: bone morphogenetic protein-15

BTC: beta-cellulin

cAMP: cyclic adenosine monophosphate

CB1: cannabinoid receptors type 1

CB2: cannabinoid receptors type 2

CBC: cannabichromene

CBD: cannabidiol

CBG: cannabigerol

CBL: cannabicyclol

CBN: cannabinol

CBRs: cannabinoid receptors

CCs: cumulus cells

CDCA8: cell division cycle-associated protein 8

CDK1: cyclin-dependent kinase 1

cDNA: complementary DNA

CENT1: centrin-1

CEP: centrosomal protein

cGMP: cyclic guanosine monophosphate

CIP2A: cancerous inhibitor of protein phosphatase 2A

CL: corpus luteum

CNS: the central nervous system

COX-2: cyclooxygenase-2

CPC: chromosomal passenger complex

CREB: cAMP-response element-binding protein

Ctr: control

CX-37: connexin-37

CX-43: connexin-43

Cy-3: cyanine 3 bisacid

CYP: cytochrome P450

CYP11A1: cytochrome P450 family 11 subfamily A member 1

CYP17A1: cytochrome P450 family 17 subfamily A member 1

CYP19A1: cytochrome P450 family 19 subfamily A member 1

DAGL: sn-1 diacylglycerol-lipases

DFs: dominant follicles

DHEA: dehydroepiandrosterone

E_2: estradiol

eCBs: endocannabinoids

ECS: endocannabinoid system

EET-EA: epoxyeicosatrienoyl-ethanolamides

EGFRs: EGF-like factors can bind their receptors

EMT: endocannabinoid membrane transporter

ER: estrogen receptors

EREG: epiregulin

FAAH: fatty acid amide hydrolase

FABP: fatty acid binding protein

FAK: focal adhesion kinase

FLAT: FAAH-like anandamide transporter

FLIP: flice-like inhibitory protein

FOXO3a: forkhead box O-3a

FSH: follicle-stimulating hormone

FSHRs: FSH receptors

GABA: gamma-aminobutyric acid

GCs: granulosa cells

GDF-9: growth differentiation factor-9

GJA1: gap junction α-1

GnRH: gonadotropin-releasing hormone

GPR: G-protein coupled receptor

GPR55: G protein-coupled receptor 55

GTP: guanosine-5'-triphosphate

GV: germinal vesicle

GVBD: germinal vesicle breakdown

HASPIN: histone H3 associated protein kinase

hCG: human chorionic gonadotropin

HPG: the hypotalamic-pituitary-gonadal axis

HPO: hypotalamic-pituitary-ovarian axis

HRP: horseradish peroxidase

Hsp70: heat shock protein 70

i.p.: intraperitoneally

ICM: inner cell mass

IFNτ: interferon τ

INCENP: inner centromere protein

IVF: in vitro fertilization

IVM: in vitro maturation

JNK: c-Jun N-terminal kinase

KIF5A: kinesin heavy chain isoform 5A

KIT: receptor-type protein-tyrosine kinase

KITL: KIT ligand

KO: knock-out

LH: luteinizing hormone

LHR: LH receptors

LOXs: lipoxygenases

LRH1: liver receptor homolog 1

MAGL: monoacylglycerol lipase

MAPK: mitogen-activated protein kinase

MCAK: mitotic centromere-associated kinesin

MEM-HEPES: hepes-buffered Eagle’s minimal essential medium

mGCs: mural GCs

MI: metaphase I

MII: metaphase II

miRs: microRNAs

ML193: ML193 trifluoroacetate

MPF: maturation promoting factor

MTOCs: microtubule-organizing centers

NAPE-PLD: N-acylphosphatidyl ethanolamines-specific phospholipase D

NC: negative controls

nd: not detectable

NEDD1: neural precursor cell expressed developmentally down-regulated protein 1

NO: nitric oxide

NOS: nitric oxide synthase

NPPC: natriuretic peptide precursor type C

NPR2: natriuretic peptide receptor 2

nSMase: neutral sphingomyelinase

NTs: neurotrophins

NuMA: nuclear mitotic apparatus protein

OCCs: oocyte-cumulus cell complexes

OEA: N-oleoylethanolamine

OSE: ovarian surface epithelium

OTCs: outer trophoblastic cells

P_4: progesterone

PBI: polar body I

PBI: polar body II

PCNT: pericentrin

PCOS: polycystic ovary syndrome

PDE3A: phosphodiesterase 3A

PDE5: phosphodiesterase 5

PEA: N-palmitoylethanolamine

PFs: primary follicles

PGCs: primordial germ cells

PI3K: phosphoinositide 3-kinase

PKA: protein kinase A

PKA: protein kinase A

PLA2: phospholipase A2

PMSG: pregnant mare serum gonadotropin

PMSG: pregnant mare serum gonadotropin

POF: premature ovarian failure

PPARs: peroxisome proliferator-activated receptors

PreAFs: preantral follicles

pre-GCs: pre-granulosa cells

PrFs: primordial follicles

PrOF: preovulatory follicle

PRs: progesterone receptors

PTEN: phosphatase and tensin homolog

RAN: Ras-related nuclear protein

RCC1: regulator of chromosome condensation 1

r.t.: room temperature

SF1: steroidogenic factor 1

SR1: SR141716A

SR2: SR144528

StAR: steroidogenic acute regulatory protein

STMN1: stathmin

TCs: theca cells

TGF: transforming growth factor

THC: Δ9-tetrahydrocannabinol

TPX2: targeting protein for Xenopus kinesin-like protein Xklp2

TRPV1: transient receptor potential vanilloid type 1

TSP1: thrombospondin 1

TUBG1: γ-tubulin

TuRC: tubulin ring complex

VEGF-A: vascular endothelial growth factor A

VEGFR2: VEGF receptor 2

XIAP: X-linked inhibitor of apoptosis

ZF: zona-free

ZP: zona pellucida

αMEM: MEM-α modification

Δ9-THCV: Δ9-tetrahydrocannabivarin

ΔΔCt: Delta–Delta Ct

Chapter 1

Background: female fertility/infertility, cannabis and the correlation between

1.1. Oogenesis and folliculogenesis: an overview

Folliculogenesis is the process that allows the development of a fully grown and fertilizable oocyte, enclosed in the complex structure of the ovarian follicle. Folliculogenesis undergoes many steps of maturation (summarized at the end of this section in Figure 1) that occur during the early embryogenesis until the end of the reproductive life in the females' adulthood.

1.1.1. Primordial germ cells and primordial follicles

During the early stage of embryonic development, it is possible to identify a small cluster of alkaline phosphatase-positive cells in the mesoderm, which are the primordial germ cells (PGCs), precursors of both oocytes and spermatozoa. PGCs migrate toward the developing genital ridges (Molyneaux *et al.*, 2001), and throughout this migration they undergo a rapid proliferation with incomplete cytokinesis, forming clusters called germ cells' cysts or nests. Afterwards, they enter meiosis following an anterior to posterior wave and finally become oocytes (Menke *et al.*, 2003; Jagarlamudi and Rajkovic, 2012; Lei and Spradling, 2013). The newly formed oocytes remain arrested at diplotene stage of the first meiotic division (prophase I), better known as germinal vesicle (GV) stage (Dutta *et al.*, 2016). In mammalians, the timing in which this process occurs is species-specific. In fact, while in mice it takes place during the first days after birth, in humans, domestic animals and primates it occurs during fetal life (Møllgård *et al.*, 2010). The breakdown of germ cell cysts is necessary for the formation of primordial follicles (PrFs), a process accompanied by a massive germ cells lost (Pepling and Spradling, 2001; Menke *et al.*, 2003). PrFs are composed of a single layer of squamous somatic pre-granulosa cells (pre-GCs), which surround one GV stage oocyte. All the PrFs constitute the ovarian quiescent follicle reserve. In waves, they can be recruited for entering in the growing pool (initial recruitment), that will continue the follicular maturation to primordial follicles (PFs) and onward. It is important to point out that the number of PrFs,

as well as the number of oocytes included in them, is fixed in early life (some days after birth for mice, during fetal life for humans), and processes of neo-oogenesis/new formation of PrFs are excluded (Porras-Gómez and Moreno-Mendoza, 2017).

1.1.2. Initial recruitment: PrFs activation and development to PF stage (gonadotropin-independent maturation)

Three are the possible destinies of PrFs: i. to rest in a quiescent state, ii. to undergo activation joining the growing pool, iii. to undergo atresia during the quiescent state (Zheng *et al.*, 2012). The most of PrFs will follow the first destiny, being kept quiescent mostly by inhibitory signals derived from the oocyte, while a selected subpopulation of few PrFs will be activated to proceed with the next phases of folliculogenesis. The morphological change of granulosa cells (GCs) from squamous and flattened to cuboidal markedly characterizes the transition from PrFs to PFs, together with the initial growth of the GV oocytes (Pedersen and Peters, 1968).

Among the oocyte-dependent signaling pathways involved in the suppression of PrFs activation, it is noteworthy to mention the role of the phosphoinositide 3-kinase (PI3K) pathway as a key regulator of this process. In fact, the loss of phosphatase and tensin homolog gene (*Pten*), as well as the deletion or hyper-phosphorylation status of Forkhead box O-3a (FOXO3a), negatively affected the regulation of PrFs activation, leading to premature ovarian failure (POF) (John *et al.*, 2008; Reddy *et al.*, 2008; Adhikari *et al.*, 2009).

On the other hand, also the activation and subsequent development of PrFs in PFs are regulated by several factors and signaling pathways, as confirmed mainly by the use of knock-out (KO) mice.

Among the numerous pathways involved, several members of the Transforming growth factor (TGF)-β family cover a pivotal role. Specifically, auto-/paracrine regulation of germ cell proliferation has been connected to the activin A (Martins da Silva *et al.*, 2004; Coutts *et al.*, 2008), together with an increased number of PrFs depending on its expression and function (Ding *et al.*, 2010; McLaughlin and Telfer, 2010; Knight *et al.*, 2012). Another paracrine factor, which inhibits in a dose-dependent manner PrFs assembly, is the anti-Müllerian hormone (AMH) (Nilsson *et al.*, 2011). In *AMH*-KO mice it has been recorded a significant depletion of PrFs in comparison with wild-type animals (Durlinger *et al.*, 1999; 2002a; 2002b). This finding has been confirmed also in humans

and in cultured rat ovaries (Carlsson *et al.*, 2006; Nilsson *et al.*, 2007). It is important to underline also the pivotal role in this mechanism of other two main TGF-β family members, as the growth differentiation factor-9 (GDF-9) and the bone morphogenetic protein-15 (BMP-15), both secreted by the oocyte. In cultured human ovarian tissues, GDF-9 promoted the activation of PrFs, as well as in goat, hamster and rat ovaries (Vitt *et al.*, 2000; Hreinsson *et al.*, 2002; Wang and Roy, 2004; Martins *et al.*, 2008). Confirming these findings, experiments with *Gdf-9*-KO mice evidenced a blockade in follicular development at the primary stage (Carabatsos *et al.*, 1998). BMP-15 stimulates FSH-independent proliferation, but its depletion affects differently the initial follicular recruitment, depending on the species. In fact, while *Bmp-15*-KO mice results subfertile, for the reduction of ovulation and fertilization rates (Yan *et al.*, 2001), sheeps with the homozygotic loss of this gene are infertile, due to insufficient PFs after PrFs activation (Galloway *et al.*, 2000).

Additionally, also the receptor-type protein-tyrosine kinase (KIT) and its ligand (stem cell factor or KITL) play an important role in PrFs activation. In fact, while the receptor KIT is expressed on the oocytes' membrane in mouse, rat, and human species, the production of KITL occurs in the pre-GCs (Tuck *et al.*, 2015). The signaling regulated by KIT/KITL is essential during the first stages of follicular development, before the FSH responsiveness. In 1997, a mouse *in vivo* study demonstrated the role of KIT in the PrFs activation, as well as in other stages of follicular development (Yoshida *et al.*, 1997). Likewise, the use of animal models lacking the KIT/KITL genes reported sterility as the main outcome due to oocyte and follicular development defects (Kuroda *et al.*, 1988; Flanagan *et al.*, 1990; Huang *et al.*, 1993). Moreover, during *in vitro* ovarian culture, the inhibition of KIT/KITL signaling induced a decrease in cyst breakdown together with increased oocyte number and reduction in cell death (Jones and Pepling, 2013). In addition, KITL action has been connected to PI3K and mitogen-activated protein kinase (MAPK) signaling, leading to the conclusion that the cyst breakdown could be mediated by those pathways in a KITL-dependent manner (Jones and Pepling, 2013).

1.1.3. Transition from PFs to preantral follicles (PreAFs) (gonadotropin-independent follicle maturation)

As the follicular development proceeds up to the secondary (preantral) stage, characterized by the GCs proliferation in two or more layers, also the enclosed oocyte continues its growth. Furthermore, a basement membrane surrounding the outer layer of GCs separates this last one from a newly formed additional layer of somatic undifferentiated cells, called theca cells (TCs). Despite being gonadotropin independent, this stage of folliculogenesis requires a complex bidirectional interaction between GCs and the growing oocyte, which finely regulates GCs and TCs proliferation and differentiation and, in the same time, needs the support of these somatic cells for its development (Edson *et al.*, 2009; Liu *et al.*, 2015). Besides, from this stage onward GCs express FSH receptors, that allow this follicle to be FSH responsive (McGee and Hsueh, 2000).

Signals responsible for the transition and the correct growth and development belong mainly to the TGF-β family (Harlow *et al.*, 2002; Pangas and Matzuk 2004; Ingman and Robertson 2009; Knight *et al.*, 2012; Matzuk and Burns 2012). Members of this family are recognized for the activation of SMAD signaling through different types of receptors. In particular, BMPs can activate SMAD-1, -5, -8, while activins, TGF-β and GFD-9 are responsible for the activation of SMAD-2, -3 (Chang *et al.*, 2002; Myers *et al.*, 2009). The different compartments of PreAFs produce several members of this family. Specifically, activins are produced by GCs, BMP-4 and -7 by TCs, TGF-β by both GCs and TCs, GDF-9 and BMP-15 by the oocyte (Oktem and Urman, 2010). It is important to remember that some of the fore-mentioned factors could be more or less important depending on the species. In fact, as stated before, most of the timings and regulation of folliculogenesis is species-specific.

The transition from PFs to PreAFs is regulated also by neurotrophins (NTs), specifically by neurotrophin-5 (NTF-5) and brain-derived neurotrophic factor (BDNF). In fact, double KO mice for *Ntf5* and *Bdnf* showed a prominent decrease in PreAF formation (Edson *et al.*, 2009).

Besides the abovementioned paracrine signaling, the transition to PreAFs and the PreAF growth itself are strictly dependent on the direct cell-to-cell communication through the formation of gap junctions between oocyte and GCs. In this context, the connexin family, peculiarly connexin-43 (CX-43) and connexin-37 (CX-37), plays a

pivotal role (Kidder and Mhawi, 2002). CX-43 and CX-37 are differently expressed in the follicular compartments. In fact, CX-43 forms gap junctions between GCs, whereas CX-37 has been found restricted to oocyte-GCs interface (Kidder and Mhawi, 2002).

Finally, the growing oocyte initiates the secretion of glycoproteins, i.e. ZP-1, -2 and -3, that altogether will constitute the zona pellucida (ZP). The somatic compartment of the follicle (GCs and TCs) contributes to the production of ZPs in a species-specific manner. Indeed, mouse oocytes synthesizes all ZPs independently (Bleil and Wassarman, 1988), while humans or large mammals need the contribution of both the oocyte and the somatic cells (Sinowatz *et al.*, 2001). Furthermore, as follicular development proceeds, TCs start to differentiate and to form two layers: i. theca interna, which secretes androgens, ii. theca externa composed of smooth muscle cells and collagen fibers (Macchiarelli *et al.*, 1992). The differentiation of TCs is accompanied by the expression of luteinizing hormone (LH) receptors (Edson *et al.*, 2009). Lastly, it is important to mention that the TCs compartment, especially the theca interna, is the only one irrorated by capillaries and blood vessels (Young and McNeilly, 2010). A correct vascularization is essential for the supply of endocrine factors to the growing follicle and can determine its fate, as for proceeding in the follicular maturation or undergo atresia (Young and McNeilly, 2010).

1.1.4. Antral stage of folliculogenesis: gonadotropin-dependent maturation

During the final stage of follicular development, the appearance of a fluid-filled cavity named antrum occurs, together with the differentiation of GCs cells in two functionally distinct population: the mural GCs (mGCs) in charge of steroidogenesis, and the cumulus cells (CCs) lining the oocyte ZP (Edson *et al.*, 2009). These follicles are named antral follicles (AFs).

The process leading to the activation and maturation of fully-grown AFs (named as Graafian follicles) begins after puberty and is connected to the increase of FSH levels during every reproductive cycle. These hormonal waves permit the rescuing of a cohort of AFs from atresia. During the cyclic recruitment, only a few follicles survive. The oocytes enclosed in these follicles have completed their growth, together with the acquisition of the ZP, and are already competent for meiosis resumption (Tsafriri, 1997; Trounson *et al.*, 1998).

The peculiarity of this complex machinery relies on the sharp separation of the different roles of these two hormones inside the population of GCs. In fact, FSH receptors

(FSHRs) are expressed only in GCs, while LH receptors (LHRs) can be found only in TCs. This model is known as "2-cell/2-gonadotropin" system.

FSH is necessary for the AFs survival, the proliferation of GCs, together with the expression of LH receptor (LHR) and the production of the sex hormone estradiol (E_2). To pursue its roles, FSH needs to bind FSHR, which is a $G_{\alpha s}$-protein coupled receptor and can activate the effector adenylyl cyclase (AC) leading to the production of the second messenger cyclic adenosine monophosphate (cAMP) and to the activation of the downstream protein kinase A (PKA) pathway. PKA activation through cAMP results in the phosphorylation of the transcription factor cAMP-response element-binding protein (CREB), therefore upregulating the expression of genes, as aromatase and LH receptor (Stocco, 2008). Additionally, FSH together with E_2 modulates GCs cell cycle promoting their proliferation (Edson *et al.*, 2009).

i. 2-cell/2-gonadotropin system: how steroidogenesis controls folliculogenesis

TCs produce androgens starting from cholesterol. Among the several enzymes are involved in this process, a key role is played by the steroidogenic acute regulatory protein (StAR), which transports cholesterol to the inner mitochondrial membrane, the cytochrome P450 family 11 subfamily A member 1 (CYP11A1), which transforms cholesterol in pregnenolone, the cytochrome P450 family 17 subfamily A member 1 (CYP17A1), which converts pregnenolone to dehydroepiandrosterone (DHEA), and the 3β-hydroxysteroid dehydrogenase (3β-HSD), which lastly converts DHEA into androstenedione. All the fore-mentioned enzymes are regulated by LH (Magoffin, 2005).

Conversely, the enzymes required for the production of E_2 starting from androstenedione are expressed by GCs. The key enzymes for this mechanism are the cytochrome P450 family 19 subfamily A member 1 (CYP19A1, also known as aromatase) and the 17β-hydroxysteroid dehydrogenase (17β-HSD). These enzymes work under the control of FSH (Magoffin, 2005).

Based on this fine and complex regulation, it is important to mention that the majority of preantral/antral follicles belonging to the growing pool will undergo atresia. In fact, among the AFs only a subcategory of this population will reach the preovulatory stage and will be able to respond to the LH surge, eventually undergoing ovulation (Salomon *et al.*, 1977). These follicles belong to the class of the so-called dominant follicles (DFs).

1.1.5. Selection of the DF

The DF is characterized by the presence of higher levels of E_2 compared to the other AFs, concluding in a higher efficiency to cause a negative feedback on FSH production and circulation. In addition, DF presents more GCs, thus a higher number of FSHR respect to the other AFs (van Santbrink *et al.*, 1995). FSH stimulation induces the expression in GCs of anti-apoptotic factors, as the X-linked inhibitor of apoptosis (XIAP) and the flice-like inhibitory protein (FLIP), while when absent, GCs release the death receptor FAS and FAS ligand (Jiang *et al.*, 2003). Moreover, DF's E_2 production moderates the FSH-dependent expression of LHR by GCs. In contrast with the other AFs, which show LHRs only on the membranes of TCs, DF acquires major responsiveness to LH through the expression of LHRs even on GCs. In addition, the mechanism of DF selection seems to be orchestrated by the development of a richer vascularization pattern, allowing a major exposure to gonadotropins.

In this matter, during follicular development, the AFs acquire a two-capillary vascular network restricted to the theca interna and externa compartment. During each cycle, a large number of AFs establish a vascular bed essential for sustaining their metabolic requirements (Brown and Russell, 2014). Yet, the newly recruited blood vessels are not able to permeate the GCs layers because of the presence of a basement membrane juxtaposed between GCs and TCs. After the ovulation of the mature oocyte and before the formation of the corpus luteum (CL), the basement membrane overthrow, permitting the pouring of blood vessels in the GCs compartment and causing massive angiogenic processes.

During DF selection, several known pro- and anti-angiogenic factors take part in this process, like Vascular endothelial growth factor A (VEGF-A), PI3K/protein kinase B (also known as Akt) pathway and Thrombospondin 1 (TSP1). The hormonal stimulation during follicular growth induces hypoxia in the follicular microenviroment, causing an increase in the expression of VEGF-A (Neeman *et al.*, 1997). Animal studies confirm this process since the injection of VEGFA in rat ovaries provoked an increase in AFs number (Danforth *et al.*, 2003) and in ovulating oocytes (Iijima *et al.*, 2005). Besides, the VEGF receptor 2 (VEGFR2) blockade caused a decrease in PreAFs formation (Zimmermann *et al.*, 2003). Moreover, in mice *Cd36*-KO, the main TSP1 receptor, it has been found a hypervascularization of the whole ovary, together with VEGF and VEGFR2

overexpression, increase in preovulatory follicles number and decrease in CL formation (Osz *et al.*, 2014).

Recently, the upregulation of peculiar microRNAs (miRs), like miR-144, miR-202, and miR-873, have been demonstrated in healthy follicles respect to the atretic ones (Tesfaye *et al.*, 2018). Moreover, it has been demonstrated that miR-21 and three different miRNA clusters (miR-183-96-182, miR-132-212, and miR-424-450-542) are mainly expressed in GCs of the preovulatory DFs, suggestingtheir involvement in rescuing GCs from apoptosis only in the DF (Carletti *et al.*, 2010; Gebremedhn *et al.*, 2016).

1.1.6. Ovulation

After the selection of one or more (depending on species) DF (that from now and on will be named as preovulatory follicle, PrOF) occurs the ovulation. In the PrOF, the FSH-stimulated GCs produce elevated levels of E_2, which acts on the hypothalamus/pituitary/ovary feedback system by increasing the pulses of the hypothalamic gonadotropin-releasing hormone (GnRH) and consequently triggering the LH surge (Richards and Pangas, 2010).

In this complex multistep mechanism, several transcriptional regulators activated in an LH-dependent fashion are involved. Firstly, it is induced the expression of progesterone receptors (PRs) in the GCs of the PrOF, which stimulate the production of cytokines (Robker *et al.*, 2000). Additionally, other transcriptional regulators are known to intercede in the ovulatory response after LH surge, including C/EBP, the nuclear receptor NR5A2 also known as liver receptor homolog 1 (LRH1), and NR5A1 also known as steroidogenic factor 1 (SF1) (Edson *et al.*, 2009).

LHR is a G-protein coupled receptor, which in GCs activates the AC and cAMP/PKA pathway. As well, in this compartment, the binding of this receptor with its ligand, LH, can promote other signaling cascades pivotal for the process of ovulation, as PI3K/Akt and RAS (Richards and Pangas, 2010). Regarding the cAMP/PKA pathway in GCs, the main downstream regulation involved the expression of the EGF-like factors amphiregulin (AREG), beta-cellulin (BTC), and epiregulin (EREG). After proteolytic cleavage, EGF-like factors can bind their receptors (EGFRs), stimulating the ERK1/2 pathway, and thus inducing the expression of target genes involved in processes like cumulus expansion, steroidogenesis, oocyte maturation, and finally ovulation. In mice, it

has been demonstrated that the disruption of the EGF pathway could impair cumulus expansion and inhibit ovulation (Hsieh *et al.*, 2007).

In conclusion, the LH ovulatory surge triggers i. oocyte meiotic resumption, ii. mucification and consequent expansion of the CCs, iii. PrOF's outer layers rupture, and iv. release of a complex constituted by a fertilizable oocyte and CCs (MII arrested oocyte surrounded by cumulus corona radiata cells). After the release of the oocyte, GCs and TCs undergo luteinization, which will lead to the formation of the corpus luteum (CL), the main producer of progesterone (P_4) (Edson *et al.*, 2009; Richards and Pangas, 2010).

1.1.7. Oocyte meiotic resumption

From the formation of PrFs until LH surge and ovulation, all the follicles contain a meiotically arrested oocyte, which is characterized by a large roundish nucleus named germinal vesicle (GV). During folliculogenesis and during the reproductive lifespan of females, most of the oocytes remain in GV stage, yet undergoing a process of growth and nuclear/cytoplasmic competence acquisition. Firstly, GVs will resume meiosis in response to LH surge through the nuclear envelope collapse and nucleolus disappearance, process better known with the term germinal vesicle breakdown (GVBD). The timings between the LH signal and the first sign of meiotic resumption and the number of oocytes that will be activated are species-specific (e.g. in mice GVBD will start after ~2-3 hours after LH, while in humans after ~18 hours) (Jaffe and Egbert, 2017). After GVBD, the oocytes will complete the first stage of meiosis (MI), evidenced by the extrusion of a small cell containing one set of chromosomes, the first polar body (PBI). The extrusion of PBI allows the oocyte to pass from a diploid (2N, in GV) to a haploid (N, in MI) chromosome arrangement, necessary at the time of fertilization. Immediately after, the MI stage oocyte will move forward to the second meiotic metaphase (MII). During MII, the remaining chromosomes will assemble on the metaphase plate of the second meiotic spindle but will arrest the division until fertilization. The MII stage oocytes, ready to be fertilized by sperm, will be extruded from follicles and will be captured by oviductal fimbria, entering in the infundibulum. Upon fertilization, the meiotic process will proceed again, causing the separation of sister chromatids and the formation of the second polar body (PBII) (Tsafriri and Dekel, 2010).

The arrest in prophase I of fully-grown GV is imposed by the presence of low levels of cyclin-dependent kinase 1 (CDK1), which is a component of the maturation promoting

factor (MPF) together with cyclin B. After CDK1 reach a sufficient amount and is activated by the removal of the inhibitory phosphate, it combines with cyclin B in the MPF complex leading to the GVBD through nuclear laminas disaggregation, formation of nuclear pores and chromosomes condensation (Adhikari *et al.*, 2012; Lénárt *et al.*, 2003; Abe *et al.*, 2011). CDK1 activity is finely regulated by high concentrations of cAMP, produced mainly by the oocyte (through the G protein-coupled receptor GPR3 and its effector AC3) and to a lesser extent by the granulosa compartment (Bornslaeger and Schultz, 1985; Horner *et al.*, 2003). The function of cAMP is exploited through the protein kinase A (PKA), which activates the kinase WEE1B for the phosphorylation of CDK1 in the inhibitory site, plus inhibits the phosphatase CDC25B to avoid CDK1 activation (Duckworth *et al.*, 2002; Han *et al.*, 2005).

In order to maintain high concentrations of cAMP, it is essential to prevent its degradation by the phosphodiesterase 3A (PDE3A). The inhibition of this enzyme is ensured by the cyclic guanosine monophosphate (cGMP), which is produced by the CCs and supplied to the oocyte through gap junctions (Norris *et al.*, 2009). The enzyme responsible for the synthesis of cGMP is the guanylyl cyclase natriuretic peptide receptor 2 (NPR2), which is activated by the natriuretic peptide precursor type C (NPPC) produced by mGCs (Zhang *et al.*, 2010; Zhang and Xia, 2012). These findings explain the pivotal role of GCs in maintaining the GV stage oocyte arrested at prophase I. In fact, it has been demonstrated that, if the oocyte is removed from the follicular microenvironment, the meiosis resumes spontaneously (Edwards, 1965).

When LH binds PrOF, it provokes the closure of gap junctions, avoiding the entrance of cGMP into the oocyte. In the meanwhile, LH decreases the production of NPPC and increase the expression of EGFR, which contributes in lowering cGMP concentration (Park *et al.*, 2004; Kawamura *et al.*, 2011; Richani and Gilchrist, 2017). In addition, the LH signal activates the downstream pathway of mGCs PKA, which is responsible for the phosphorylation of phosphodiesterase 5 (PDE5) and, consequently, the degradation in CCs of cGMP (Egbert *et al.*, 2016). Thus, the reduction of cGMP levels together with the closure of gap junctions result in a sharp fall in cAMP levels inside the oocyte (through PDE3A), in the inactivation of oocyte PKA signaling, which inhibits no more CDC25B. Active CDC25B can dephosphorylate CDK1, allowing the formation of the MPF complex and the resumption of meiosis (Adhikari and Liu, 2014).

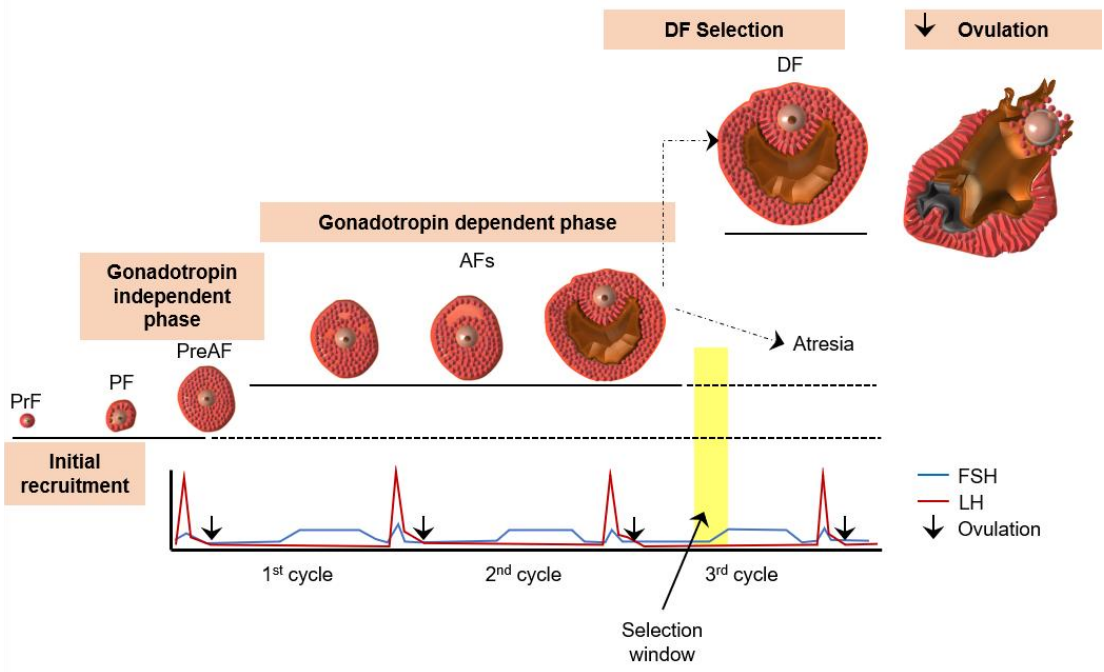


Figure 1. Schematic representation of all stages of folliculogenesis until ovulation.
Abbreviations: AFs, antral follicles; DF, dominant follicle; FSH, follicle stimulating hormone; LH, luteinizing hormone; PF, primary follicle; PreAF, preantral follicle; PrF, primordial follicle.

1.1.8. Meiotic spindle organization during the first (I) and second (II) meiotic division

After oocyte meiotic resumption, the female gametes assemble two spindles to assure chromosome segregation and the formation of a haploid egg ready for fertilization (Ohkura, 2015). Despite similarities, meiotic spindle formation is subjected to species-specific regulation (Figure 2).

Differently from mitotic cells, oocytes miss centrosomes as microtubule-organizing centers (MTOCs) (Bennabi *et al.*, 2016). However, bipolar spindles can be assembled without these structures, due to the existence of two mechanisms identified in MI and MII spindle assembly, the chromosome- and the acentriolar MTOC-mediated microtubule formation. An important adaptative advantage of the small and anastral meiotic spindles is the asymmetric divisions and the small size of PB, reducing the frequency of aneuploidy due to incorrect separation (Cortes *et al.*, 2015).

Concerning the chromosome-mediated microtubule formation, the predominant role is covered by the small GTPase Ras-related nuclear protein (RAN), which is involved in nuclear transport and nuclear biochemical processes' regulation (Clarke and Zhang, 2008). The active form of this GTPase is dependent on the chromatin bounded regulator of chromosome condensation 1 (RCC1), which creates a gradient of RAN-GTP starting from the chromatin (Kalab *et al.*, 2002). This gradient provokes the activation of factors

necessary for spindle assembly, as the targeting protein for Xenopus kinesin-like protein Xklp2 (TPX2) (Gruss *et al.*, 2002; Prosser and Pelletier, 2017). Among its targets, Aurora-A protein kinase (AURKA) and the tubulin ring complex (TuRC) start microtubule nucleation at spindle poles. This mechanism is indispensable in human oocytes MI spindle formation (Holubcová *et al.*, 2017), while it is necessary for mouse oocytes but only in combination with the MTOC-mediated mechanisms (Dumont *et al.*, 2007).

Besides RAN-GTP mechanism, a key role for chromatin-mediated microtubule nucleation is played by the chromosomal passenger complex (CPC) (Carmena *et al.*, 2012). This complex involves the inner centromere protein (INCENP), the Aurora-B protein kinase (AURKB), the baculoviral IAP repeat-containing protein 5 (BIRC5, better known as survivin) and the cell division cycle-associated protein 8 (CDCA8, or Borealin). Starting from the chromatin, CPC creates a gradient of AURKB (Fuller *et al.*, 2008), which in turn inactivates the mitotic centromere-associated kinesin (MCAK) (Lan *et al.*, 2004) and the stathmin (STMN1) (Gadea and Ruderman, 2006) through phosphorylation. Thus, CPC induces the nucleation of microtubules at the kinetochore (Prosser and Pelletier, 2017). Together with AURKA and AURKB, also the Aurora C protein kinase (AURKC) is essential for meiotic spindle correct assembly (Balboula and Schindler, 2014). In fact, the perturbation of this kinase or of its regulator (histone H3 associated protein kinase, HASPIN) reduces MTOCs clustering at the final part of spindles (Balboula *et al.*, 2016).

Regarding acentriolar MTOC-mediated microtubule formation, the exact molecular organization is not yet fully clarified. Commonly to the mitotic centrosome, acentriolar MTOCs include pericentrin (PCNT) (Carabatsos *et al.*, 2000), γ-tubulin (TUBG1) (Gueth-Hallonet *et al.*, 1993), neural precursor cell expressed developmentally down-regulated protein 1 (NEDD1) (Ma *et al.*, 2010), centrin-1 (CENT1) (Manandhar *et al.*, 2006), nuclear mitotic apparatus protein (NuMA) (Lee *et al.*, 2000), cancerous inhibitor of protein phosphatase 2A protein (CIP2A) (Wang *et al.*, 2017), and the centrosomal proteins CEP192 and CEP152 (Lee *et al.*, 2018). Throughout mouse oocyte maturation, acentriolar MTOCs spatial organization changes. In GV-stage mouse oocytes, MTOCs surround the GV in large clusters (Clift and Schuh, 2015). Before GVBD, MTOCs move towards the entire nuclear membrane, while after GVBD they are organized in small foci, from which starts the microtubule nucleation. Finally, during mouse oocyte maturation, MTOCs undergo self-organization to both spindle's ends, allowing the formation of a

bipolar spindle, together with the kinesin heavy chain isoform 5A (KIF5A) and the dynein (Clift and Schuh, 2015).

The importance of understanding the mechanisms underlying oocyte meiotic spindle formation is based on the fact that the aberrant assembly of a meiotic spindle could lead to aneuploidies. Overall, the spindle position, dimensions, and shape are essential markers of oocyte developmental potential (Eichenlaub-Ritter *et al.*, 2004).

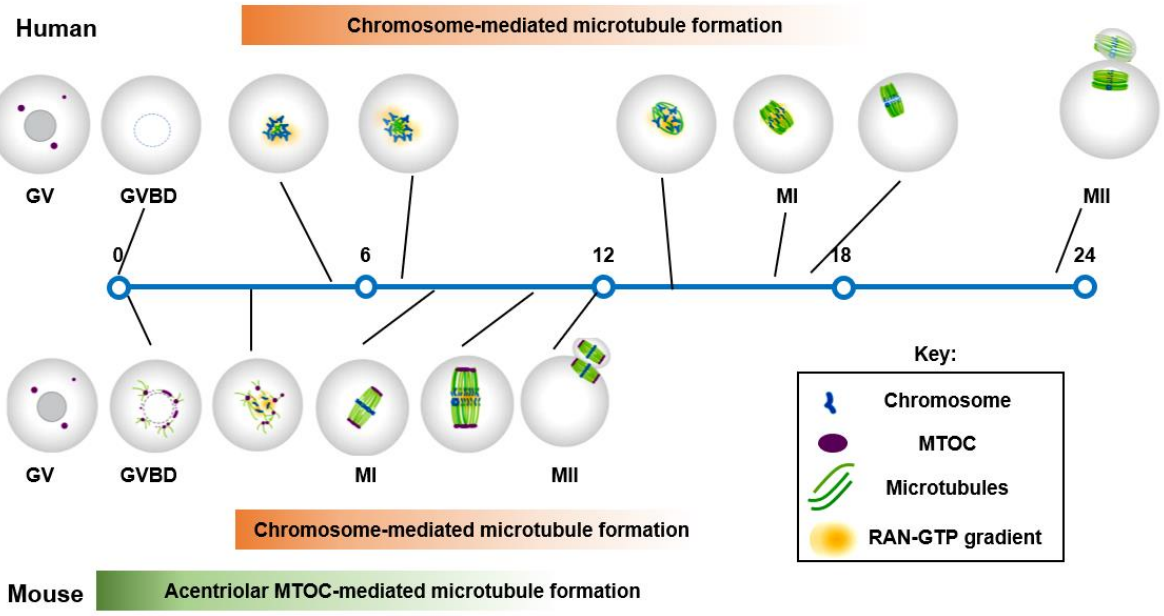


Figure 2. Schematic representation of timings and mechanisms involved in spindle formation in human and mouse oocytes (modified by Namgoong and Kim, 2018).
Abbreviations: GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; MII, metaphase II; MTOC, microtubule-organizing centers; RAN, GTPase Ras-related nuclear protein; GTP, guanosine-5'-triphosphate.

1.2. The issue of female infertility

The ultimate aim of folliculogenesis is to produce fertilizable oocytes. Thus, the physiological progression of this clockwork process ensures female fertility. Nowadays, the issue that the world is facing is the failure of this process, moving the balance needle up to infertility. The western world is getting older. In fact, the majority of the developed countries show a constant decrease in fertility rate. The highest fertility rate belongs to Africa (Niger: 7,153 children/woman, Somalia: 6,123 children/woman), a phenomenon related to the lower social status and the need for children as workforce. Likewise, socio-economic factors (e.g. lack of affordable housing, flexible and part-time jobs) strongly contribute to the current low fertility/birth rates. In fact, although densely populated, the

highly industrialized China and India have fertility rates comparable to those of European countries and USA, ranging from 1.88 to 1.22 children/woman (http://worldpopulationreview.com/countries/total-fertility-rate/).

The declining birth rate is influenced by several elements. First, women's lifestyle choices force them unconditionally to postpone motherhood, determining a significant increase in the population of reproductively aged women. Indeed, the chance of conceiving per month is halved by age 35, and it drops to 1% by age 45 (Broekmans *et al.*, 2007). However, reproductively aged women seem to underestimate the negative effect of older age in which such a decline occurs, or the age-dependent increased risk of obstetric complications (Lockwood, 2011). On the other hand, women firmly rely, sometimes overestimating, on the performances of assisted reproductive technologies (ART) (Fritz and Jindal, 2018).

Many other causes, as anovulation (Weiss and Clapauch, 2014), POF (Vander Borght and Wyns, 2018; Huang *et al.*, 2019), polycystic ovary syndrome (PCOS) (Vander Borght and Wyns, 2018; Broughton and Moley, 2017), endometriosis (Vander Borght and Wyns, 2018; Tomassetti and D'Hooghe, 2018) and the whole range of treatments with high gonadotoxic potential, as anticancer therapies (Vander Borght and Wyns, 2018), have been demonstrated to dramatically reduce women's reproductive lifespan.

Concerning cancer, even if it does not impair directly fertility (Das *et al*., 2011; Lefebvre *et al.*, 2018), it has an indirect effect worth to mention. In fact, the most common anticancer therapies, that are chemotherapy and radiotherapy, have massive gonadotoxic side effects, resulting in an iatrogenic loss of fertility in cancer affected patients (Salama *et al.*, 2017). Particularly, the majority of gonadotoxic drugs inhibit folliculogenesis in different stages: e.g. small follicles appear to be irrevocably damaged by alkylating agents such as cyclophosphamide (Falcone *et al.*, 2004). In addition, gonadotoxic treatments can negatively alter the number of ovarian follicles causing maturation deficits in terms of cycle arrest of actively dividing cells or high toxicity on primordial follicles, the normal formation of capillaries and can cause cortical fibrosis. If persistent in time, this reduction in the number and quality of follicles can possibly lead to POF and, subsequently, early menopause (De Vos *et al.*, 2010). As well, radiotherapy has a dose- and age-dependent effect on the ovary (Howell and Shalet, 1998). Like chemotherapy gonadotoxic treatments, radiations impair rapidly dividing cells in follicles undergoing maturation and the quiescent primordial follicles pool (Meirow and Nugent, 2001).

Impairment of fecundity has to face also air pollutants, pesticides, and chemicals present in water and food. Indeed, many of them can act as endocrine disruptors, promoting oxidative stress and exerting genotoxic effects through the formation of DNA adducts and/or epigenetic modifications (Carré *et al.*, 2017). These endocrine-disrupting properties are exerted by interacting either with estrogen or androgen receptors (ER and AR, respectively) or with specific targets activating the Ras/Erk pathway and inflammatory processes. While the effects of air pollutants on human spermatogenesis have been extensively investigated (Jurewicz *et al.*, 2009; Hammoud *et al.*, 2010; de Angelis *et al.*, 2017), those on female fertility are still a matter of debate, even if from a recent study it seems that air pollution could represent a matter of concern also for women fecundity, particularly regarding increased miscarriage rate and reduced IVF fertilization rates (Conforti *et al.*, 2018). On the other hand, about hazard of other chemicals as bisphenol A (used in the production of polycarbonate plastics and phthalate plasticizers) (Rashtian *et al.*, 2019), and the pesticides mancozeb and lindane, both widely used in agriculture, experimental data clearly demonstrated that they can compromise ovarian functions by reducing the production of fertilizable oocytes (Rossi *et al.*, 2006; Palmerini *et al.*, 2017; 2018).

From all this data, it is evident that infertility is a multifactorial pathology and that the decline in women's fertility is due mainly to ovarian aging as well as to several other factors, leading to reduced chance of conception.

Which measures can be taken to prevent it? A new approach named "ecofertility", that requires improvement in the lifestyle, early diagnosis and proper therapies of the pathologies impairing fertility, should be adopted (Alvarez, 2015). Moreover, strategies aimed to ensure fertility to young oncologic patients subjected to gonadotoxic therapies should be applied. In fact, the need to undergo such deleterious but most of the time lifesaving therapies can affect permanently the female reproductive capacity (Levine *et al.*, 2015; Algarroba *et al.*, 2018; Medrano *et al.*, 2018). To date, different options can be offered to such patients, as storage of gametes, embryos or the cryopreservation of either whole ovary or cortical fragments (strips) of the ovarian tissue followed by transplantation (Rossi *et al.*, 2019).

In this wide range context of infertility, it is worth to mention also how the use of addictive substances (as alcohol, cigarette smoke, and drugs) can contribute to this new reproductive pathology (Fody and Walker, 1985; Emanuele *et al.*, 2002; Weiss and Clapauch, 2014; Vander Borght and Wyns, 2018). In particular, alcohol has a well-known

teratogenic effect (Vander Borght and Wyns, 2018), while it has been demonstrated that the cigarette smoke contains heavy metals, polycyclic hydrocarbons, nitrosamines, and aromatic amines, all of which have a detrimental effect on health and on fertility itself (Dechanet *et al.*, 2011). Recreational drug misuse seems to affect all reproductive events. In men, it has been demonstrated that the repetitive long-term cannabis consumption causes a decrease in sperm liquid volume and in spermatozoa concentration, affecting their morphology and motility, increasing sperm hyperactivity and, thus, reducing their fertilization capacity (Carrol *et al.*, 2020). In women, observational studies have linked cannabis use to dysregulated menstrual cycle, reduced number of oocytes obtained during in vitro fertilization (IVF) techniques and higher risk of preterm labor and birth (Wang *et al.*, 2006a). In this complex picture, the wide use of drugs requires a special mention.

1.3. Recreational use and abuse of cannabinoids in female fertility and pregnancy

Due to the increase in permissive legal and social environments of developed countries, as Australia, New Zealand, Spain, United Kingdom, Italy, the Netherlands, Canada, and more than 20 States in the USA, marijuana has become one of the most consumed illicit (and sometimes licit) recreational drugs (https://www.therecoveryvillage.com/marijuana-addiction/). What happened worldwide in the last years is the legalization of cannabis for medical use and the decriminalization for the possession of "non-criminal offense" for recreational use (summarized in Figure 3). Recently, in Europe, several countries - including Ireland (Flanagan, 2013), Poland (Palikot Movement, 2013), France (Benbassa, 2014), Portugal (Bloco de Esquerda, 2015), Germany (Terpe *et al.*, 2015), the Netherlands (Tweede Kamer der Staten-Generaal, 2015) and Italy (Giachetti *et al.*, 2015) - faced the proposal of legislative models to join cannabis legalization from politician leading the national parliament. An example closer to the Italian reality shows that statistically prevalence in Italy reached 9.95, meaning that almost 10 out of 1,000 Italians are drug users, or are suffering from drug use disorders/dependence (https://www.statista.com/topics/3823/drug-situation-in-europe/).

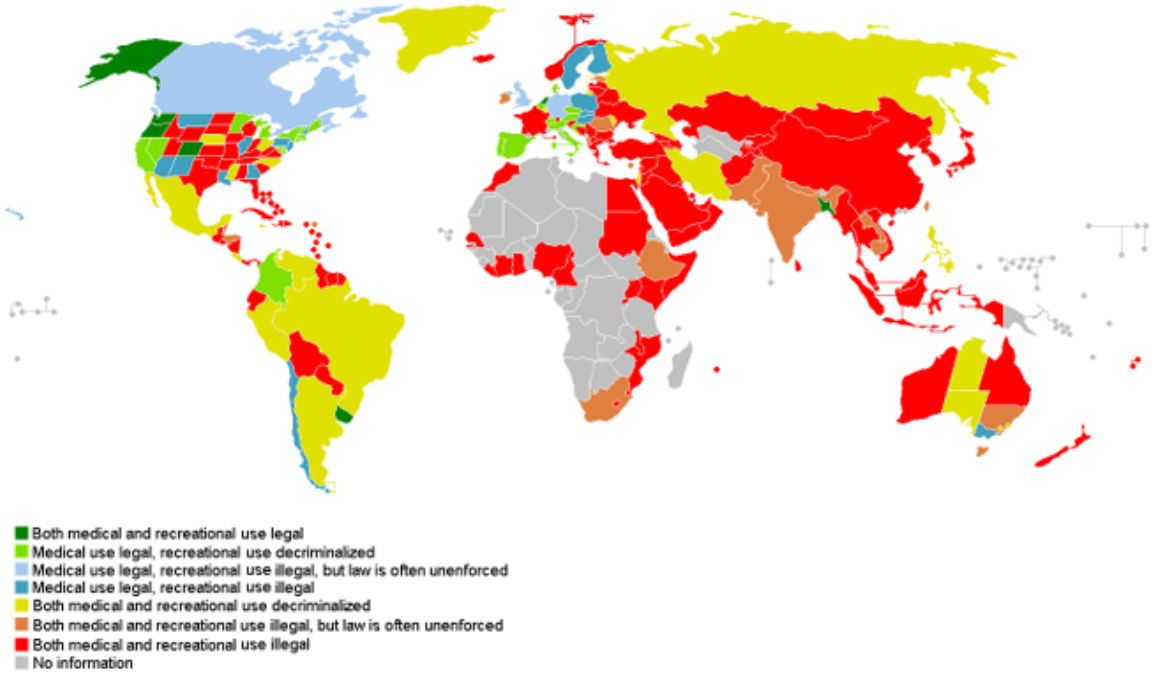


Figure 3. World map of the legalization of cannabis for medical and recreational use and of unenforced law/decriminalization for the possession of "non-criminal offense" recreational use (taken by the free copyright website: Wikipedia, SCG Legal counsel, Country website).

Besides its medical purpose still to properly estimate at clinical level, the increasing use of cannabis in a recreational mode among young women prioritizes the need to consider its side effects in female reproduction. Indeed, the negative impact of cannabis consumption has raised numerous warnings about the possible damage for the reproductive potential, particularly concerning pregnancy and the long-term outcomes of newborns (Hayatbakhsh *et al.*, 2011). Specifically, more than 9% of women consume regularly marijuana before pregnancy, while 2.5% continue during pregnancy (Hayatbakhsh *et al.*, 2011). One of the biggest issues of marijuana use is the young women's misperception of this drug as a "no risk" consumption, thus underestimating the potential risks of its use even only once or twice per week (Ko *et al.*, 2015). The use of marijuana has been evaluated in several epidemiologic studies, from which controversial results emerged about the negative side effects of cannabis' maternal use in pregnancy outcomes (Shiono *et al.*, 1995; Fergusson *et al.*, 2002).

Δ^9-tetrahydrocannabinol (THC) enters the maternal circulation and can easily pass through the placental membrane (Marchetti *et al.*, 2017). For this reason, gestational disorders as implantation failure, preterm birth, intrauterine growth restriction, low birth weight and increased risk of miscarriage have been correlated with natural and synthetic cannabinoids use during pregnancy, together with an abnormal embryo development and

the occurrence of tubal pregnancies (Sun and Dey, 2014; Varner *et al.*, 2014; Richardson *et al.*, 2016; Metz *et al.*, 2017). Yet, cannabis use during pregnancy was associated with an increased risk of stillbirth as demonstrated by the presence of THC in the umbilical cord homogenate (Varner *et al.*, 2014). The association with neonatal morbidity could be due to long-term modifications in the immunologic response at the time of cannabinoids exposure in the uterus (Zumbrun *et al.*, 2015; Metz *et al.*, 2017). On the other hand, other studies on cannabis consumption during the period of pregnancy did not find any association with abnormal fetal growth (Metz and Stickrath, 2015), but only a tendency in increased risk for defect in congenital birth associated with cannabis use during early stages of pregnancy (Merlob *et al.*, 2017). As far, it is worth to mention that many synthetic cannabinoids (also called as cannabimimetic compounds) show a higher affinity to the THC-binding cellular receptors, thereby resulting in a more effective negative impact against pregnancy (Huffman and Padgett, 2005). As it is evident in this complex scenario, there are several discrepancies among different studies, due to the use of different methodologies for the quantification of the amount of marijuana consumed and to the evaluation of a heterogeneous gamma of biological samples, such as plasma, urine, and umbilical cord homogenates.

Taking a step forward pregnancy and discussing the "just-become" mothers, also the use of marijuana during the lactation period has been studied. Worryingly, it has been discovered that cannabis and its metabolites are highly detected in breast milk, depending on maternal consumption. Regular cannabis consumers during breastfeeding reported an 8-fold higher THC concentration in human milk in comparison with maternal serum concentration (Jaques *et al.*, 2004; Belendiuk *et al.*, 2015). The overall limitation of these studies resides in the lack of long-term follow-up on newborns, who were unwillingly exposed to this kind of drug.

Chapter 2

The truth on the endocannabinoid system (ECS): what is it and why is it important to know it?

2.1. Cannabinoids, endocannabinoids (eCBs) and ECS

Cannabis plants (particularly *Cannabis sativa* and *Cannabis indica*) contain more than 545 different lipophilic chemical messengers (phytocannabinoids), of which over 100 have been identified so far, including the first most potent psychoactive Δ^9-THC. Phytocannabinoids are a family of lipophilic terpeno-phenolic compounds and are classified as neutral cannabinoids (without carboxyl group) and cannabinoid acids (with carboxyl group) (Hanuš *et al.*, 2016). In *Cannabis sativa*, the main detected cannabinoids, besides Δ^9-THC, are cannabidiol (CBD), cannabigerol (CBG), Δ^9-tetrahydrocannabivarin (Δ^9-THCV), cannabichromene (CBC), cannabicyclol (CBL), cannabinol (CBN), which all are biosynthesized and accumulated as cannabinoid acids, and subsequently decarboxylated into their neutral forms (Bonini *et al.*, 2018).

Recent investigations suggest the use of cannabinoid-based drugs for a wide range of medical conditions, including neurological and psychiatric disorders, pain, anorexia and the relief of chemotherapy side-effects (Whiting *et al.*, 2015; Amato *et al.*, 2017; Fraguas-Sánchez and Torres-Suárez, 2018). For instance, the non-psychoactive phytocannabinoid CBD has been suggested as a new therapeutic agent in Alzheimer's disease, Parkinson's disease, cancer, and infertility (Laun *et al.*, 2018), although clinical data do not completely support its use (Whiting *et al.*, 2015; EMCDDA, 2018; Friedman *et al.*, 2019).

Except for the phytocannabinoids and the synthetic compounds above mentioned, the cannabinoid family includes also a class of different fatty acid-derived lipids synthesized by our organism, named endocannabinoids (eCBs, Table 1). The first hint to the existence of eCBs was in the early 1990s when it was discovered an organism endogenous system capable of mimicking the effects of the Δ^9-THC, through the interaction with the same cannabinoid receptors (CBRs) (Devane *et al.*, 1992, Mechoulam *et al.*, 1995, Sugiura *et al.*, 1995). The first endogenous compound was discovered in 1992 and has it has been named as *N*- arachidonoylethanolamine (AEA), widely known as anandamide, (Devane *et al.*, 1992). Later, in 1995 the second most important eCB was isolated and named as

monoacylglycerol 2-arachidonoylglycerol (2-AG) (Mechoulam *et al.*, 1995). Since 1992 and on, several studies investigated the presence and the functions of eCBs in different species. In 2002, Sugiura and collaborators detected the presence of eCBs mainly in the brain and in other several organs and body fluids (Sugiura *et al.*, 2002), finding a general 100/200-fold lower concentration of AEA in comparison with 2-AG.

Other molecules have been proposed to belong to the eCB family, including 2-AG-ether (noladin ether) and *O*-arachidonoylethanolamine (virodhamine). Among these "eCB-like" compounds, two additional *N*-acylethanolamines, *N*-palmitoylethanolamine (PEA) and *N*-oleoylethanolamine (OEA), have been extensively investigated for their anti-inflammatory and analgesic properties. Differently from AEA and 2-AG, PEA and OEA are considered inactive on CBRs and it has been hypothesized that they could act through modulating AEA binding to CBRs, which results in the inhibition of its metabolic degradation and in the prolongation of its biological effect. This mechanism of action is called "entourage effect" (Ho *et al.*, 2008; Maccarrone and Alexander, 2012).

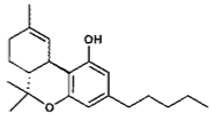
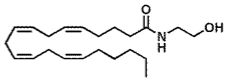
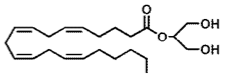
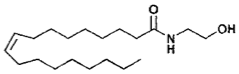
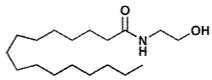
Endocannabinoids (eCBs)	Chemical structure
Δ^9-Tetrahydrocannabinol (THC)	
N-Arachidonoylethanolamine (Anandamide, AEA)	
2-Arachidonoylglycerol (2-AG)	
eCB-like compounds	**Chemical structure**
N-Oleoylethanolamine (OEA)	
N-Palmitoylethanolamine (PEA)	
Metabolic enzymes of AEA	**Intracellular localization**
N-acylphosphatidyl ethanolamines (NAPE)-specific phospholipase D (NAPE-PLD)	Membrane-associated
Fatty acid amide hydrolase (FAAH)	Membrane-associated (mainly ER)
Metabolic enzymes of 2-AG	**Intracellular localization**
Diacylglycerol lipase α (DAGLα)	Membrane-associated
Diacylglycerol lipase β (DAGLβ)	Membrane-associated
Monoacylglycerol lipase (MAGL)	Membrane-associated and cytosolic
α/β-Hydrolase domain protein 6 (ABHD6)	Membrane-associated enzyme

Table 1. Major eCBs and eCB-like compounds chemical structure, and main metabolic enzymes of the ECS with the respective intracellular localization (modified by Cecconi *et al.*, 2019)

All these endogenous compounds represent the neuro-/immuno-modulators and peripheral signals that can control various physiological and pathological processes, like cardiovascular, nervous, immune system and reproductive functions, inside the organism by interacting within a regulatory and ubiquitous pro-homeostatic system named endocannabinoid system (ECS, Figure 4) (Maccarrone *et al.*, 2015; Maccarrone, 2017; Rapino *et al.*, 2018). The effects of cannabinoids and eCBs occur through binding to

specific receptors of ECS (CBRs), which includes also metabolic enzymes responsible for eCBs' synthesis and degradation and proteins involved in the transport of eCBs inside the cell. Similar to their relative compounds phytocannabinoids, eCB-based drugs have been developed as palliative-treatment for women's reproductive pain, e.g. creams containing PEA, which appear useful as a remedy for chronic pelvic and vaginal pains (Hesselink and Hekker, 2012; Gabrielsson *et al.*, 2016). In addition, an association between micronized PEA and transpolydatin, a derivate polyphenolic compound, was effective in the management of pelvic pain related to endometriosis after laparoscopy (Cobellis *et al.*, 2011).

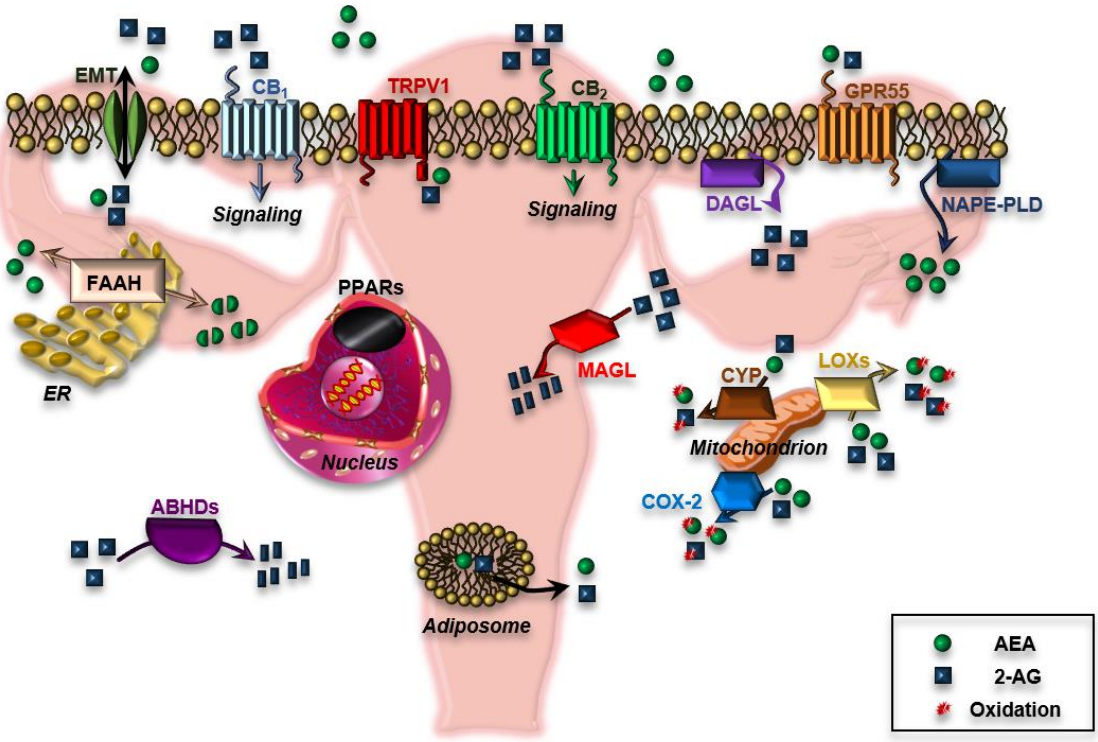


Figure 4. Cellular distribution ECS component (modified by Cecconi *et al.*, 2019). Abbreviations: 2-AG, 2-arachidonoylglycerol; ABHDs, α/β-hydrolase domain protein; AEA, *N*-arachidonoylethanolamine; CB_1, type 1 cannabinoid receptor; CB_2, type 2 cannabinoid receptor; CYP, cytochrome P_{450}; DAGL, *sn*-1 diacylglycerol-lipase; EMT, endocannabinoid membrane transporter; FAAH, fatty acid amide hydrolase; GPR55, G protein-coupled receptor 55; LOXs, lipoxygenases; COX-2, cyclooxygenase-2; MAGL, monoacylglycerol lipase; NAPE-PLD, N-acylphosphatydil ethanolamines (NAPE)-specific phospholipase D; PPAR, peroxisome proliferator activated receptor; TRPV1, transient receptor potential vanilloid-1.

2.2. eCBs-target receptors (CBRs) and downstream signaling

The discovery of CBRs initiated in 1990 when Matsuda and collaborators using a rat model succeeded in isolating and cloning the membrane receptor responsible for binding Δ^9-THC: the type-1 cannabinoid receptors (CB_1) (Matsuda *et al.*, 1990). Later in 1993, Munro and colleagues discovered the type-2 cannabinoid receptors (CB_2) (Munro *et al.*,

1993). During the last years of the XX century, several scientists investigated the localization of these CBRs, finding out that CB_1 is expressed preferentially in the brain (Herkenham *et al.*, 1991) and, in a lesser extent, in peripheral organs (Galiegue *et al.*, 1995; Kenney *et al.*, 1999), while CB_2 can be found mainly in immune system tissues (Parolaro *et al.*, 2002). Besides CB_1 and CB_2, another CBR is the so-called "orphan" G protein-coupled receptor 55 (GPR55, also known as CB_3) (Lauckner *et al.*, 2008; Maccarrone and Alexander, 2012; Gasperi *et al.*, 2013), which it has been found also in ovarian and prostatic cancer cells, confirming a potential role in tumor proliferation (Moriconi *et al.*, 2010).

All these CBRs are G protein-coupled receptors and, upon activation by eCBs, they can activate a manifold range of intracellular signaling (the main signaling activated by each receptor are summarized in Table 2), depending on the G protein to them connected ($G_{\alpha s}$, $G_{\alpha i}$, or G_q). Some intracellular signaling activated by eCBs are the following:

- inhibition of AC through $G_{\alpha i}$, with consequent reduction of cAMP levels and silencing of PKA (Brighton *et al.*, 2011);
- activation of MAPK and ERK, direct or through modulation of β-arrestin (Brighton *et al.*, 2011; Ibsen *et al.*, 2017);
- inhibition of voltage-dependent Ca^{2+} channels type L, N, P/Q (Mato *et al.*, 2009) and T (Gilmore *et al.*, 2012);
- activation of voltage-dependent K^+ channels (Mato *et al.*, 2009);
- activation of focal adhesion kinase (FAK), p38 and c-Jun N-terminal kinase (JNK) (Gómez *et al.*, 2014);
- activation of the enzyme nitric oxide synthase (NOS), leading to consequent production and release of NO (Gratzke *et al.*, 2010);
- activation of cytosolic phospholipase A2 (PLA2) (Signorello *et al.*, 2011);
- activation of the neutral sphingomyelinase (nSMase) to produce ceramide (Giuliano *et al.*, 2006);
- activation of the serine palmitoyltransferase for *de novo* synthesis of ceramide (Kang *et al.*, 2010).

CBRs	Main Ligands	Main Functions
CB_1	AEA, 2-AG	AC inhibition
CB_2	2-AG	AC inhibition Ca_i^{2+} release
GPR55	PEA, OEA, AEA, 2-AG	Ca_i^{2+} release
TRPV1	AEA	$>Ca^{2+}$ influx Regulation of cationic currents

Table 2. The main functions of the major CBRs activated by the main ligands. Abbreviations: 2-AG, 2-arachidonoylglycerol; AC, adenylyl cyclase; AEA, *N*-arachidonoylethanolamine; Ca_i^{2+}, intracellular calcium; CB_1, type 1 cannabinoid receptor; CB_2, type 2 cannabinoid receptor; CBRs, cannabinoid receptors; GPR55, G protein-coupled receptor 55; OEA, *N*-oleoylethanolamine; PEA, *N*-palmitoylethanolamine; TRPV1, transient receptor potential vanilloid-1.

At the same time, eCBs can interact with other types of non-GPRs receptors, like the transient receptor potential vanilloid type-1 (TRPV1) and the nuclear peroxisome proliferator-activated receptors (PPARs). TRPV1 is a non-selective cationic channel consisting of six transmembrane domains with the N- and C-terminals protruding into the cytosol (Pertwee *et al.*, 2010), which provides the entrance of mono- or divalent cations (preferentially Ca^{2+}) upon AEA/heat/acid pH activation (Ryskamp *et al.*, 2014). Concerning PPARs, they belong to the family of nuclear receptors involved in the regulation of lipid and glucidic metabolism and they are divided into three subtypes: PPARα, PPARδ, and PPARγ. PPARα is naturally activated by OEA and PEA, while AEA and 2-AG are responsible for the activation of PPARγ (Pertwee *et al.*, 2010; Farce *et al.*, 2009).

2.3. eCBs metabolic enzymes and transporters

The activity of eCBs is controlled by their endogenous levels, i.e. the balance between the mechanisms of synthesis and degradation. Previous studies promoted the theory of eCB-synthesis and release “on demand”, even if recently it has been advanced the hypothesis that eCBs can be stored in reservoirs, such as adiposomes (Oddi *et al*., 2008; Maccarrone *et al.*, 2010). This smart choice of storage serves as a potential platform for eCBs trafficking and accumulation, allowing also the prolongation of their half-life (hours rather than minutes) (Maccarrone *et al.*, 2010).

This fine balance depends on the functionality of different enzymes. For what it concerns AEA, the *N*-acylphosphatidyl ethanolamines (NAPE)-specific phospholipase D (NAPE-PLD) is in charge of the biosynthesis of this eCB (Okamoto *et al.*, 2009), which is degraded by the fatty acid amide hydrolase (FAAH) type-1 or -2 (FAAH-1, FAAH-2) (McKinney and Cravatt, 2005). Regarding 2-AG, it is mainly synthesized by the *sn*-1 diacylglycerol-lipases (DAGL)-α (in the central nervous system) and -β (in peripheral organs) (Bisogno *et al.*, 2003), while it is degraded by monoacylglycerol lipase (MAGL) (Dinh *et al.*, 2002). Furthermore, the regulation of intracellular 2-AG levels depends on enzymes belonging to the serine hydrolase family: α/β-hydrolase domain protein-6 and -12 (ABHD6, ABHD12) (Blankman *et al.*, 2007). Inter alia, the regulation of ABHD6 seems to be controlled by hormones, like E2, implying that its expression could be sex dependent (Drehmer *et al.*, 2019).

An alternative destiny of AEA and 2-AG, apart from degradation, is the oxidative metabolism (Rouzer and Marnett, 2011). These eCBs can be oxidized by cyclooxygenase-2 (COX-2), different lipoxygenases (LOXs) or cytochrome P_{450} (CYP) (Van der Stelt *et al.*, 2002; Snider *et al.*, 2010; Rouzer and Marnett, 2011). LOXs convert eCBs into conjugated hydroperoxides, rapidly reduced in the corresponding hydroxides. Inside the cell, several isoforms of this enzyme are detected, among which the most common are 12-LOX and 15-LOX, which convert the AEA into the FAAH inhibitors 12-HAEA and 15-HAEA, respectively (Amadio *et al.*, 2010). On the other hand, 2-AG is converted by 15-LOX into 15-HETE-G (Kozak *et al.*, 2002). Similarly, COX-2 is involved in the production of prostanoids from arachidonic acid(Alhouayek and Muccioli, 2014). Lastly, CYP converts AEA into several epoxyeicosatrienoyl-ethanolamides (EET-EA) (Yang *et al.*, 2011).

It is worth to mention also that all eCBs can be inactivated through the process of uptake. To date, several mechanisms of cellular uptake have been described: i. facilitated transport (Fezza *et al.*, 2008), ii. passive diffusion regulated by the catabolic enzyme FAAH (Kaczocha *et al.*, 2006), iii. passive diffusion regulated by intracellular sequestration (Oddi *et al.*, 2008), iv. passive diffusion regulated by the formation of AEA-cholesterol complexes (Di Pasquale *et al.*, 2009), v. caveola-dependent endocytosis (Fowler, 2013). Both AEA and 2-AG are recovered by the cell through a selective, saturable transporter temperature-dependent and Na^+-independent, known as anandamide membrane transporter (AMT), or more generally, "endocannabinoid membrane transporter" (EMT), whose structure and molecular identity remains yet to be identified

(Chicca *et al.*, 2017). To date, the functioning of the general AEA transport mechanism is still under debate

In this context, different cytoplasmic eCB-binding proteins have been identified in support of cellular uptake mechanisms (Chicca et al., 2012). Namely, constitutive intracellular transporters include the heat shock protein 70 (Hsp70) and albumin (Oddi et al., 2009), as well as fatty acid binding protein-5 and -7 (FABP5 and FABP7) (Kaczocha et al., 2012) and the FAAH-like anandamide transporter (FLAT) (Leung et al., 2013). More recently, potent and selective EC uptake inhibitors (WOBE437 and RX-055) have been extensively profiled to target eCBs membrane trafficking (Chicca et al., 2017).

Chapter 3
ECS and female reproduction in mammals

The different functions that ECS exerts in the female reproduction have been investigated by numerous studies on animal models and humans, through which it has been proved the presence of ECS elements and eCBs in several reproductive fluids and tissues, e.g. human serum and follicular fluid, and mammalian ovary, uterus, and placenta (El-Talatini *et al.*, 2009a, 2009b; Trabucco *et al.*, 2009; Taylor *et al.*, 2010a; Fonseca *et al.*, 2012; Ding *et al.*, 2017; Maia *et al.*, 2017). Based on these evidences, the indirect modulation of eCBs levels in each specific reproductive tissue, through their metabolism, targeting, and trafficking, is a key-factor for female reproduction and fertility (Cecconi *et al.*, 2014; Rapino *et al.*, 2014; Walker *et al.*, 2019). For instance, a fine balance of the ECS guarantees the success of fertilization and pregnancy (Taylor *et al.*, 2010a), whilst a dysregulation of eCBs tone affects negatively various steps of female reproduction (from gametogenesis to fertilization, embryo implantation/development, and parturition), that could lead to miscarriages and pregnancy complications, such as endometriosis (Shen *et al.*, 2019), ectopic pregnancies (Gebeh *et al.*, 2012) and preeclampsia (Molvarec *et al.*, 2015).

3.1. ECS in the ovary: a species-specific approach

In the last years, literature data evidenced that the long-lasting exposure to cannabis and its active compounds can display important side effects on follicular development, mainly by reducing the release of hypotalamic-pituitary-ovarian axis (HPO) hormones and, thereby, of sex steroids (Brents, 2016). In fact, the HPO axis seems to be closely related to the ECS, due to the presence of CB_1 in the regions of hypothalamus and anterior pituitary (Gammon *et al.*, 2005), and of both CB_1 and CB_2 in the ovary (Cecconi *et al.*, 2019). The effects of cannabis derivatives on male/female reproductive processes pointed out to a potential impact of eCBs on the hypotalamic-pituitary-gonadal (HPG) axis. In females, the main outcome of cannabis consumers involved several mechanisms far or near to the ovary itself, such reduced GnRH levels in the blood circulation (Gammon *et al.*, 2005), anovulatory cycles, and prolonged follicular phase which results in ovulation delay (Jukic *et al.*, 2007). To date, the exact mechanisms underlying these hypothalamic

dysfunctions have not yet been elucidated. One of the hypotheses relies on the regulation via CB_1 of neurotransmitters, like glutamate and gamma-aminobutyric acid (GABA) (Grants *et al.*, 2018). The effects of cannabinoids through these neurotransmitters are equal and opposite: on one hand, cannabinoids reduce the activity of glutamate which promotes GnRH secretion, whereas they stimulate GABA activity which normally downregulates GnRH secretion (Ottem *et al.*, 2004; Rodríguez-Muñoz *et al.*, 2016). In this context, different studies demonstrated, by using a wide variety of CB_1 agonists, the GnRH regulation by the ECS (Chakravarty *et al.*, 1979; Scorticati *et al.*, 2004; Gammon *et al.*, 2005; Bálint *et al.*, 2016).

Focusing on the last discovery about the ovarian ECS in terms of functionality, De Domenico and collaborators found that the pharmacological activation of CB_2 through the injection of one of its most potent agonists (JWH133) caused in fetal female germ cells an acceleration of oocytes entrance in meiosis and apoptosis, resulting in reduced ovarian reserve (De Domenico *et al.*, 2017).

Considering eCBs signaling in the mammalian ovary, it has been shown a positive correlation between systemic and ovarian AEA levels (El-Talatini *et al.*, 2009b). Regarding the physiological context in fertile women, follicle cells produce AEA and its concentration increases alongside follicle growth, following an AEA-dependent autocrine mechanism of GCs (Brents, 2016). The reason for the low AEA levels during the first stages of folliculogenesis is connected to the necessity of follicles to prevent the oocyte maturation before the correct timing (Walker *et al.*, 2019). In fact, the peak of follicular fluid AEA concentration is achieved in the stage before ovulation, with a value of ~1.0 nM AEA that can be useful to evaluate oocyte maturity (El-Talatini *et al.*, 2009b). As for humans, also in cattle it has been recorded a similar variation of AEA levels during the estrus cycle, with the concentration peak at the periovulatory stage (Gervasi *et al.*, 2013).

Alongside AEA fluctuation, also FAAH presents a physiological modification in its expression levels during the ovulatory cycle (Correa *et al.*, 2016). Indeed, during the periovulatory phase, AEA and E_2 high levels juxtapose FAAH lower expression, whereas during the post-ovulatory phase it happens the opposite (Brents, 2016). This mechanism is confirmed by the effect of THC, which is metabolized slower than AEA and, thus, accumulates in the adipose tissue, mimicking the condition of eCBs' excess (Karasu *et al.*, 2011). In addition, the increase of serum AEA levels is positively correlated to gonadotropins and E_2 levels, but not to P_4 (El-Talatini *et al.*, 2009b). However, no correlation between E_2 and 2-AG has been observed (Scorticati *et al.*, 2004).

On the basis of these observations, despite the difficulties of extrapolating a general model of ECS ovarian functionality due to the species-specific expression and distribution, recent literature data have increased the possible roles of this system to produce healthy offspring in mammals. The information of the present section is schematically represented in Figure 5 and summarized in Table 3.

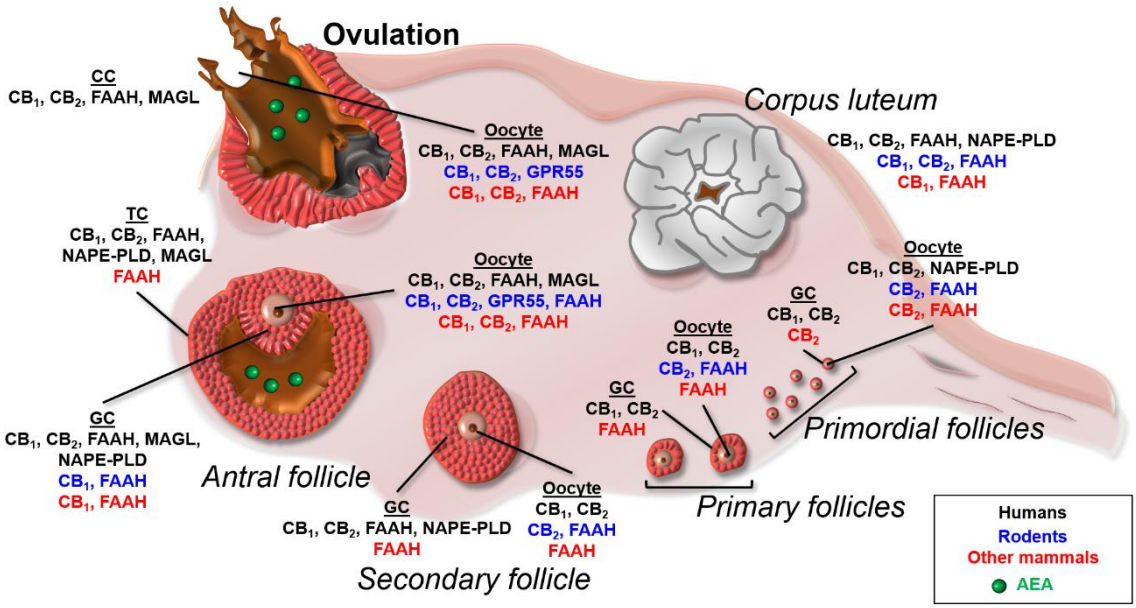


Figure 5. ECS in mammalian ovary. Each follicle is formed by the oocyte and surrounding granulosa cells (GC); theca cells (TC) layers appear at the secondary stage. The expression and localization of ECS components are modulated during folliculogenesis in a species-specific manner (black: human, blue: rodents, red: other mammals) (modified by Cecconi *et al.*, 2019). Abbreviations: AEA, N-arachidonoylethanolamine; CB_1, type 1 cannabinoid receptor; CB_2, type 2 cannabinoid receptor; CC, cumulus cells; FAAH, fatty acid amide hydrolase; GC, granulosa cells; GPR55, G protein-coupled receptor 55; MAGL, monoacylglycerol lipase; NAPE-PLD, *N*-acylphosphatydilethanolamines (NAPE)-specific phospholipase D; TC, theca cells.

3.1.1. Humans

The presence of ECS components in the human adult ovary was demonstrated for the first time by El-Talatini and collaborators through immunohistochemical analysis (El-Talatini *et al.*, 2009a). They found out the presence of CB_1 and CB_2 in oocytes of PrFs, PFs, and PreAFs follicles. After the prentral-to-antral transition, only CB_2 was detectable in oocytes. Concerning the metabolic enzymes, while NAPE-PLD was sporadically observed only in oocytes enclosed in PrFs, FAAH signal was always undetectable. As for the compartment of GCs, the immunolocalization of CB_1 and CB_2 appeared evident throughout all the stages of folliculogenesis, whilst that of FAAH and NAPE-PLD emerged only in PreAFs and sharply decreased in AFs. On the contrary, TCs always expressed CB_1, CB_2, NAPE-PLD, and FAAH. The same expression pattern was found in CL, with the exception of FAAH expression, that was undetectable after ovulation. The

conclusion of El-Talatini and coworkers was that AEA could have an autocrine signaling in TCs, and their results strengthen the hypothesis of a FAAH-independent AEA degradation in GCs (El-Talatini *et al.*, 2009a).

In human oocytes retrieved during assisted reproductive technologies (ARTs) procedures, the mRNA levels and protein expression and localization of CB_1, CB_2, MAGL, and FAAH was investigated by Agirregoitia and collaborators (Peralta *et al.*, 2011; Agirregoitia *et al.*, 2015; 2016). This research group found out that CB_1, but not CB_2, mRNA was slightly detectable during oocyte meiotic maturation. Nevertheless, both CB_1 and CB_2 were detected as proteins by Western blot and immunofluorescence analyses (Peralta *et al.*, 2011). In the same samples, FAAH mRNA and protein was expressed from GV to MII phase, whereas only MAGL protein has been found. By the use of confocal analysis, they described a peripheral distribution of both CB_1 and FAAH, while MAGL signal was cytoplasmic at GV and MI. When the oocyte reached the maturity at MII, CB_1 remained located in the membrane, while both FAAH and MAGL distribution was homogeneous inside the cytoplasm (Agirregoitia *et al.*, 2016).

The same research group performed analyses on human CCs, detecting CB_1, FAAH and MAGL at mRNA and protein level, whilst CB_2 could be observed only as a protein, mirroring the results obtained for human oocytes (Agirregoitia *et al.*, 2015). The results of these studies are not always in accordance with those obtained by El-Talatini and colleagues. This could be explained by the different methods utilized for the detection of ECS components, as by the inconsistency use from Agirregoitia and coworkers of a mixture of oocytes at different stages of maturation during the assessment of mRNAs (Real time-PCR) and proteins (Western blot) levels, which cannot be quantified in a stage-specific manner.

3.1.2. Rodents

In 2010, Bagavandoss and Grimshaw investigated for the first time the presence of some ECS components (namely CB_1, CB_2, and FAAH) in the ovaries of Sprague-Dawley rats, by using immunofluorescence and Western blot analyses (Bagavandoss *et al.*, 2010). They found that CB_2 and FAAH were present in oocytes enclosed in all the follicle maturation stages (from PrFs to AFs), while CB_1 was never expressed. In addition, CB_2 and FAAH were detected in CLs and in the ovarian surface epithelium (OSE), while no immunofluorescent signal was observed in GCs and TCs. Conversely, CB_1 is located only

in GCs of AFs, in the luteal cells after the CL formation and in OSE. The authors suggested that CB_1 expression could be due to the progressive FSH increase during follicular development, while FAAH and CB_2 protein expression was gonadotropin independent.

Different results were reported by López-Cardona and coworkers, concerning the detection of CB_1 and CB_2 in mice oocytes at different stages of meiotic maturation (López-Cardona *et al.*, 2017). In fact, they found a distinct expression of these receptors based on the stage of meiotic maturation and the protocol utilized for oocyte culture. At mRNA level, only CB_1 was detectable, while the immunofluorescence of both CBRs was recorded in the oocytes, with different cellular localization. As a matter of fact, CB_2 signal was always homogeneously distributed inside the cytoplasm, independently of the oocyte maturation stage (GV, MI or MII) and the maturation method (*in vivo* or *in vitro*). On the other hand, CB_1 localization was conditioned by *in vivo* or *in vitro* maturation: in the first case, CB_1 was homogeneously distributed in the cytoplasm of GV-stage oocytes, while it changed in peripherally distributed in MI- and MII-stages oocytes; in oocytes undergone *in vitro* maturation (IVM) CB_1 was always found at the periphery of the cell in all the maturation stages. Unfortunately, the authors did not explain either hypothesize a possible reason for these differences in the receptors' localization. However, they investigated the effect of both CBRs agonists and antagonists during the maturation process and they found out a CB_1-dependent phosphorylation of Akt and ERK1/2, respectively connected to the activation or inhibition of PBI extrusion and spindle formation. For what it concerns CB_2 presence inside the oocyte, López-Cardona and collaborators assumed a compensatory role of this receptor in case of CB_1 deletion, fundamental during the embryo transport in the oviduct (López-Cardona *et al.*, 2017).

Recently, a study from Totorikaguena and colleagues examined the effects of THC during oocyte IVM protocols in wild type C57BL/6 mice and mice KO for CB_1, CB_2 and both CB_1/CB_2 (Totorikaguena *et al.*, 2019). The main effect of THC during IVM protocols regarded the acceleration of CB_1 relocation from the cytoplasm to the periphery of the oocyte in the initial stage of maturation (GVBD), while no differences in CB_2 localization were recorded in presence or absence of this phytocannabinoid. In accordance with CB_1 results, the incubation with THC during oocyte maturation hastened the phosphorylation of ERK1/2 and, to a lesser extent, of Akt. The authors conclude that THC could be a useful supplement for IVM oocytes, in terms of better outcomes in oocyte meiotic maturation quality (by analyzing maturation kinetics, location pattern of CB_1 and

CB_2 and phosphorylation status of Akt and ERK1/2) and of increased blastocyst rate after IVF.

3.1.3. Other mammals

The ECS presence has been explored also in other mammals' ovaries, such as bovine and domestic cat. In bovine oocytes undergoing IVM, CB_1 and CB_2 mRNA levels and protein localization have been studied by López-Cardona and collaborators (López-Cardona *et al.*, 2016). Both CBRs mRNAs were observed in immature oocytes (i.e. GV and MI), whereas at MII stage only CB_2 mRNA was detected. Concerning receptor localization, the immunofluorescence analysis highlighted differences at MI and MII: in fact, CB_2 was always homogeneously located in the cytoplasm, whilst CB_1 was relocated in the periphery of the oocyte after MI stage. Concomitantly, the authors found a positive modulation by CB_1 agonists on the phosphorylation-pattern of Akt and ERK1/2, both in GCs and oocytes. In addition, CB_1 agonists positively regulated the expression of two genes known to be involved in embryo quality, namely *IFNτ* (interferon τ) and *GJA1* (gap junction α-1).

For what concerns ECS expression and distribution in the cat ovary, Pirone and collaborators (2017) investigated the presence of CB_1 and FAAH by immunohistochemistry analysis. The authors described the presence of CB_1 in AFs and, later on, in CLs, while FAAH was detected not only in both GCs and TCs of PFs, PreAFs, and AFs but also in oocyte cytoplasm and CLs. These results sustain the hypothesis that the ovarian injection of CB_1 agonists in cats could be a useful method for decreasing the fertility of this domestic animal, reducing P_4 levels.

HUMANS					
Follicle stage	**Protein**	**Oocyte**	**GC**	**TC**	**Reference**
PrF	CB_1	+	+	×	El-Talatini *et al.,* 2009a
	CB_2	++	++	×	
	FAAH	-	-	×	
	NAPE-PLD	+/-	-	×	
PF	CB_1	+	+	×	
	CB_2	++	+	×	
	FAAH	-	-	×	
	NAPE-PLD	-	-	×	
PreAF	CB_1	+	+	+	
	CB_2	++	+	+	
	FAAH	-	+	++	
	NAPE-PLD	-	++	++	
AF	CB_1	-	++	+	
	CB_2	++	+	+	
	FAAH	-	+/-	++	
	NAPE-PLD	-	+/-	+	
Corpus luteum	CB_1	×	++		
	CB_2	×	+		
	FAAH	×	+		
	NAPE-PLD	×	++		
RODENTS					
Follicle stage	**Protein**	**Oocyte**	**GC**	**TC**	**Reference**
PreAF	CB_1	-	-	-	Bagavandoss and Grimshaw, 2010
	CB_2	+	-	-	
	FAAH	+	-	-	
AF	CB_1	-	+	-	
	CB_2	+	-	-	
	FAAH	+	-	-	
Corpus luteum	CB_1	×	+		
	CB_2	×	+		
	FAAH	×	+		
CATS					
Follicle stage	**Protein**	**Oocyte**	**GC**	**TC**	**Reference**
PrF	CB_1	-	-	×	Pirone *et al.*, 2017
	FAAH	+/-	+	×	
PF	CB_1	-	-	×	
	FAAH	+/-	+	×	
PreAF	CB_1	-	-	-	
	FAAH	+	+	+/-	
AF	CB_1	+/-	+	-	
	FAAH	+	+/-	+/-	
Corpus luteum	CB_1	×	+		
	FAAH	×	++		

Table 3. Localization of ECS components in the ovary of humans, rodents, and cats. All the data are extrapolated from IHC data (modified by Cecconi *et al.*, 2019).
Notes: (-) absent; (+) present; (++) highly expressed; (+/-) occasionally observed. (x) indicates the absence of TC/oocyte. GC, granulosa cells; TC, theca cells.

3.2. ECS in the oviduct: from fertilization to early embryogenesis

The oviduct is the organ in which sperm capacitation, fertilization, embryo early development and transport to reach the site of implantation occur. Indeed, the oviduct is the structural and functional connection between the ovary and the uterus and is composed of different regions with specific functions and microenvironment molecular composition. The lower region, named isthmus, is the oviduct-uterine junction, in which uncapacitated spermatozoa get in touch with oviductal epithelial cells that induce sperm capacitation processes. After capacitation, the spermatozoa break away from the oviductal epithelium and they continue the journey up to the upper region, named ampulla, where the ovulated oocyte (MII) lies until fertilization occurs. In fact, after ovulation, the MII oocyte is caught by the fimbriae located at the beginning of the ampullary region (Li and Winuthayanon, 2017).

As mentioned before, these regions have a different molecular composition in order to fulfill the specific processes. One of these differences concerns the ECS components presence and distribution. Firstly, literature data clearly report the presence of AEA, its metabolic enzymes (NAPE-PLD, FAAH) and main receptors (CB_1, CB_2) in the oviduct, as shown in Figure 6 (Wang *et al.*, 2006b; 2006c). Interestingly, a longitudinal gradient of AEA throughout the oviduct has been found to be necessary for prolonging the fertile life of spermatozoa and for helping their advancement towards the fertilizable oocyte. Specifically, AEA concentration is higher in the isthmus, in which this eCB takes part in the regulation of sperm capacitation by releasing spermatozoa from oviductal epithelium via CB_1 and TRPV1 through the stimulation of Ca^{2+} influx (Gervasi *et al.*, 2016). Subsequently, capacitated spermatozoa secrete eCBs like AEA and 2-AG, leading to CB_1 and TRPV1 activation (Catanzaro *et al.*, 2011). As well, it has been documented that an abnormal activation of CB_1 signaling can negatively affect the interaction between sperm and oocyte during fertilization (Battista *et al.*, 2008), while the deletion of FAAH gene compromises the fertilization ability of sperm (Sun *et al.*, 2009). Studies in humans reported the role of AEA via TRPV1 signaling in the induction of sperm-egg fusion (through the inhibition of acrosome reaction and membrane fusion in sperm that are not ready for fertilization) and of oviductal and decidua CB_1 decreased expression in promoting ectopic pregnancy (Maccarrone *et al.*, 2005; Horne *et al.*, 2008; Francavilla *et al.*, 2009). Systematically, protein concentration and activity of FAAH and NAPE-PLD are equal and opposite in the different regions of the oviduct: FAAH content is high in

the ampulla and low in the isthmus, whereas the opposite situation is evident for NAPE-PLD (Scotchie *et al.*, 2015). Regarding the effect on embryo development and implantation, when maternal FAAH deficiency occurs, the high levels of AEA in the oviduct provoke embryo retention (Sun and Dey, 2012), leading to delay in its development (El-Talatini *et al.*, 2009b).

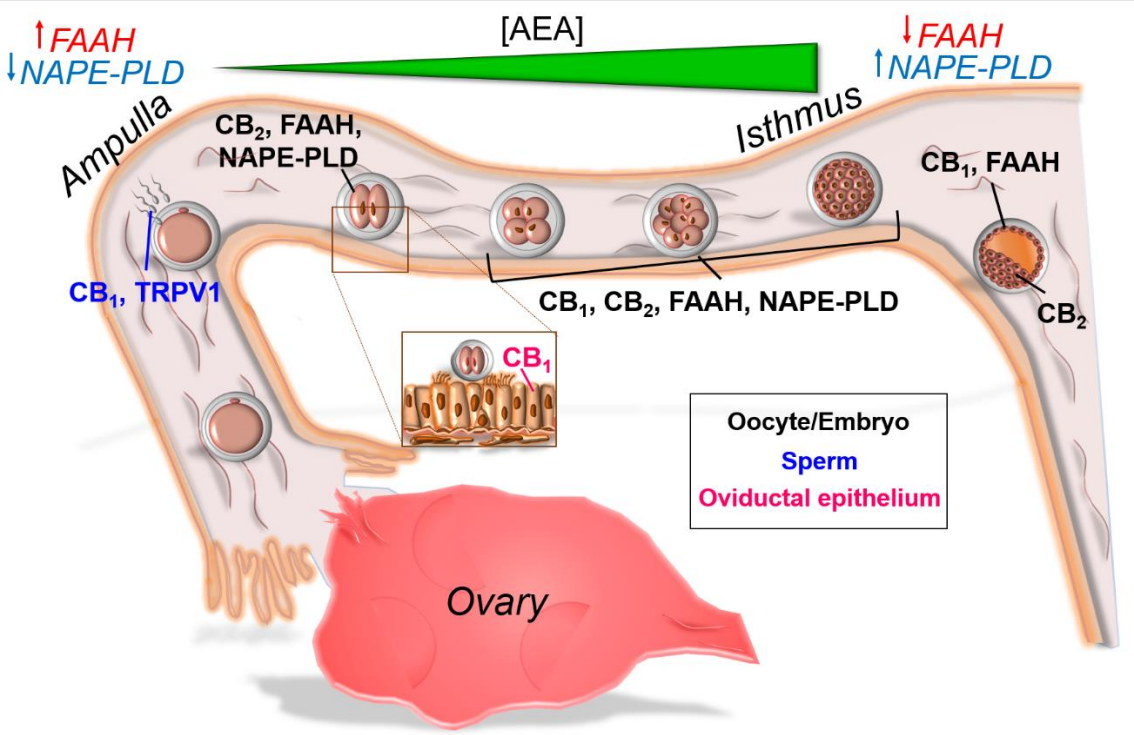


Figure 6. ECS, embryo development and oviductal transport. The expression of ECS components is modulated during fertilization, embryo development and oviductal transport (embryo: black; oviduct: green; sperm: blue). An increasing gradient of AEA is present in the oviduct from the ampulla region to the isthmus, accompanied by the modulation of AEA metabolic enzymes (modified by Cecconi *et al.*, 2019). Abbreviations: AEA, *N*-arachidonoylethanolamine; CB_1, cannabinoid receptor type 1; CB_2, cannabinoid receptor type 2; FAAH, fatty acid amide hydrolase; NAPE-PLD, *N*-acylphosphatydilethanolamines (NAPE)-specific phospholipase D; TRPV1, transient receptor potential vanilloid-1.

In this matter, it is important to discuss also the presence and the modulation of ECS in embryos. In 1995, for the first time, Paria and collaborators investigated CB_1 and CB_2 expression in mouse embryos (Paria *et al.*, 1995). They found that CB_1 is present from the 1-cell embryo stage, while CB_2 appears from the 4-cells up to the blastocyst stage. In the blastocyst, CB_2 localization is confined in the inner cell mass (ICM), while CB_1 is predominantly found in the trophectoderm (Paria *et al.*, 1995). In addition, AEA metabolic enzymes, FAAH and NAPE-PLD, are also present in the embryo, starting from the 2-cells stage up to blastocyst. In particular, FAAH was localized in the outer cells of morula and in the trophectoderm (Wang *et al.*, 2006c). In 2001, the same research group demonstrated a role of CB_1 in the regulation of embryo development and transport (Paria *et al.*, 2001). To date, the role of embryonic CB_2 remains still unknown (Maccarrone,

2009). Finally, literature data demonstrate that high levels of eCBs (AEA and 2-AG) impairs embryo development and trophoblast differentiation (Sun *et al.*, 2010).

3.3. ECS in the uterus

After fertilization and the early stages of embryogenesis, the pre-implantation embryo reaches the stage of blastocyst and, together, the final part of the isthmus from which it will fall inside the uterus. At this moment, the blastocyst will enter in contact with the uterine wall and a delicate embryo-endometrium dialog will take place. According to blastocyst competence and endometrial receptivity, the period covering 6 to 10 days in humans will be known as the "implantation window", during which the blastocyst will invade the endometrium. This process, called implantation, is characterized by the secretion of the human chorionic gonadotropin (hCG) and will continue its development.

During implantation and the downstream processes, several molecular mechanisms will occur to ensure the correct development and growth of the embryo and, later on, of the fetus. Among these mechanisms, the ECS has been proved to be involved in the physiological succession of processes, as well as its dysregulation could be the cause of pathological conditions (Correa *et al.*, 2016).

3.3.1. Implantation

In the first instance, it is noteworthy to mention that eCB signaling regulates both endometrial plasticity and uterine receptivity (Maccarrone, 2009). In fact, the fine balance between FAAH and NAPE-PLD provides the correct AEA tone, which seems to be necessary for implantation (Figure 7); on the contrary, the alteration of this eCB tuning can lead to pregnancy failure (Sun and Dey, 2009). One of the processes regulated by the ECS, through the activation of Akt and ERK1/2 pathways via CB_1, is endometrial cell migration (Gentilini *et al.*, 2010).

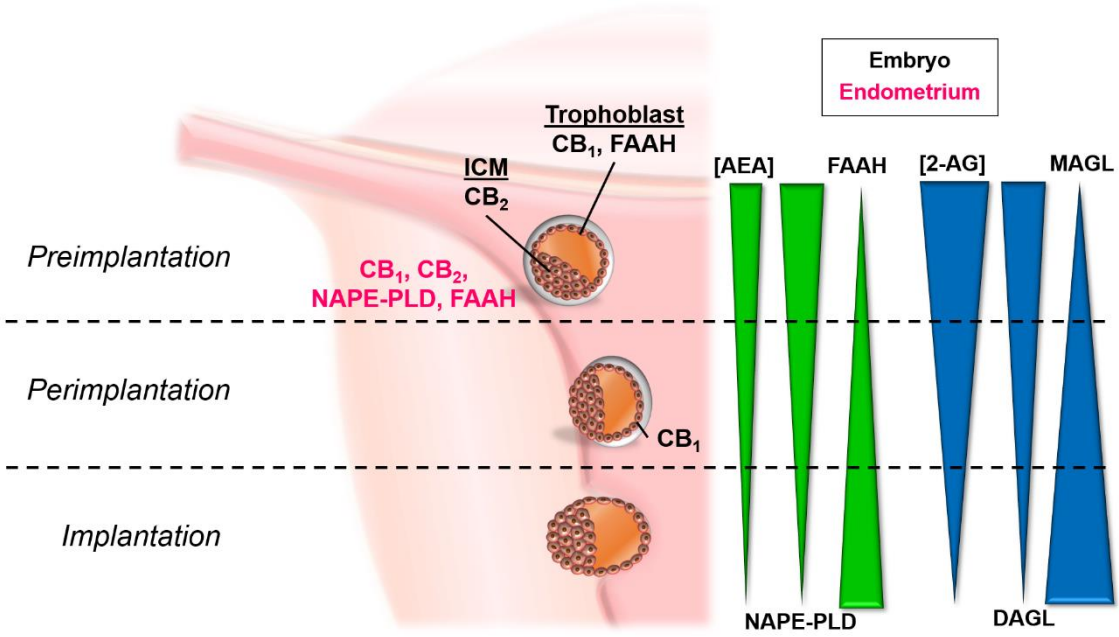


Figure 7. ECS and embryo implantation. AEA (green) and 2-AG (blue) fluctuations and relative metabolic enzymes concentrations during the process of implantation in mammals (modified by Cecconi *et al.*, 2019). Abbreviations: 2-AG, 2-arachidonoylglycerol; AEA, *N*-arachidonoylethanolamine; CB_1, type 1 cannabinoid receptor; CB_2, type 2 cannabinoid receptor; DAGL, *sn*-1 diacylglycerol-lipase; FAAH, fatty acid amide hydrolase; ICM, inner cell mass; MAGL, monoacylglycerol lipase; NAPE-PLD, *N*-acylphosphatydil ethanolamines (NAPE)-specific phospholipase D.

In this context, the use of mouse models allowed to find that the levels of the main eCBs, AEA and 2-AG, together with the expression of their metabolic enzymes are controlled by sex hormones, P_4 and E_2 (Fonseca *et al.*, 2010a; 2010b). More in details, before implantation, from day 1 to 4, the NAPE-PLD expression is preferentially detected in luminal and glandular epithelium, rather than in the stroma; whereas on days 5 to 7, its localization is restricted to the inter-implantation site (which requires high levels of AEA) and this enzyme disappear in the implantation site. As expected, the behavior of FAAH mirrors exactly the one of NAPE-PLD, finding the higher expression at day 5, coinciding with the implantation window (Sordelli *et al.*, 2012). Thanks to this fine balance, it is possible to notice a fluctuation in uterine AEA concentration, particularly the 4^{th} day of pregnancy, which is coordinated with CB_1 expression in the blastocyst (Xie *et al.*, 2012). Altogether, these data support the hypothesis that also the embryo could act as a main regulator of eCBs tone. In fact, the blastocyst during implantation produces and releases a "FAAH activator", which helps in the reduction of AEA at the site of implantation (Maccarrone *et al.*, 2004). Regarding 2-AG, it has been demonstrated that also this eCB levels (200-fold higher than AEA) are important for successful implantation. In fact, dysregulation in the mechanisms of its synthesis (DAGL-α) and degradation (MAGL and

COX-2) in the implantation site lead to an abnormal 2-AG tone, thus impairing early pregnancy events (Wang *et al.*, 2007).

In the woman's uterus, CB_2 distribution is divided between glandular and stromal tissues, being maximally expressed during the late proliferative phase. Conversely, contrasting data are available for CB_1 regulation in the uterus, together with its distribution, remaining still as an unclear matter. In fact, while Taylor and collaborators detected steroid-independent CB_1 expression mainly in the glandular epithelium (Taylor *et al.*, 2010b), Resuehr and colleagues described a P_4-dependent CB_1 expression circumscribed to the stromal tissue (Resuehr *et al.*, 2012). Furthermore, FAAH and NAPE-PLD were observed in both glandular and stromal tissues, and their expression was regulated in order to keep low AEA concentration during the mid-luteal phase.

3.3.2. Decidualization and placentation

After implantation, when the blastocyst enters in contact with endometrial stroma, a succession of events necessary for the positive outcome of early pregnancy occurs. Firstly, the uterus will undergo a process called decidualization that, under different stimuli, allows the increase in endometrial vascular permeability around the implantation site through the activation of apoptotic mechanisms in the luminal epithelium. Concomitantly, the embryo trophoectoderm will invade the uterine stroma, in order to create a niche in which it will grow, and, in the end, it will start the process of placentation (Battista *et al.*, 2015). During placentation, the blastocyst is divided into outer trophoblastic cells (OTCs) and inner cell mass (ICM), separated by an antrum, named blastocoel, filled with blastocoelic fluid (BF). The correct distribution of OTCs is mandatory for the proper placenta formation, thus defects in the physiological trophoblast differentiation can compromise placentation. In this matter, a balanced AEA level plays a pivotal role during OTCs differentiation: higher levels of this eCB negatively affect the binding activity of fibronectins, while lower levels promote MAPK signaling (Sun *et al.*, 2010), likely via CB_1 activation (Xie *et al.*, 2012).

Concerning decidualization, it has been demonstrated in rat models that higher levels of AEA, dependent on the expression of NAPE-PLD, are implicated in the process of decidua remodeling (Fonseca *et al.*, 2013; 2014). On the other hand, the activity of the AEA degrading enzyme, FAAH, is prevalent during placentation, allowing the regulation of early pregnancy events (Fonseca *et al.*, 2014).

The ECS seems to have a role during placentation also in humans. In fact, 2-AG and its metabolic enzymes, MAGL and DAGL, have been detected in the cytotrophoblast of human embryos (Habayeb *et al.*, 2008; Fonseca *et al.*, 2013), in addition to high mRNA levels of CB_1, CB_2 and FAAH in the syncytiotrophoblast during the first trimester of pregnancy and in amniotic epithelial cells (Habayeb *et al.*, 2008).

One of the most crucial periods of early pregnancy is the first trimester, during which the concomitancy of several factors can lead to early miscarriage. In this matter, a high abortion rate has been correlated with abnormal levels of CB_1, NAPE-PLD and particularly with lower nuclear FAAH expression in the trophoblast, causing higher AEA tone in the placenta (Park *et al.*, 2003; Chamley *et al.*, 2008; Trabucco *et al.*, 2009; Taylor *et al.*, 2011; Meccariello *et al.*, 2014).

3.3.3. Labor

The labor is the final step of pregnancy and, also at this moment, signals from both mother (inflammation) and fetus (hormones) collaborate in enabling parturition. The prevalent role in this signaling is covered by maternal P_4, by maintaining myometrial quiescence (Dodd and Crowther 2010; Tan *et al.*, 2012), and fetal corticotrophin hormone, by controlling the timings of gestation and delivery (Grammatopoulos, 2007). Among the several molecular mechanisms involved in the regulation of this final process, also the ECS has been described as a key regulator system.

In *Cnr1*-KO mice a higher rate of preterm birth has been described, connected to the alteration of both P_4 and corticotrophin hormone (Wang *et al.*, 2008; Sun and Dey, 2012). In women AEA rate, when reaches the peak of 1.8 nM at parturition, is responsible for regulating onset and timing of the labor (Habayeb *et al.*, 2004; Wang *et al.*, 2008). P_4 controls AEA levels through FAAH expression, having an impact also on CBRs activation of AEA (Nallendran *et al.*, 2010). In addition, in the placenta after labor, both CB_1 and FAAH are highly detected (Brents, 2016; Correa *et al.*, 2016). Furthermore, also high plasma level of the eCB-like compound, PEA, has been classified as a preterm birth biomarker (Bachkangi *et al.*, 2019).

Chapter 4

Aim of the thesis

In the evolutionary axis of life, ECS is one of the most long-lasting and phylogenetically highly preserved biological systems. Especially in mammals, its presence and functional significance for the physiological regulation of several organs and apparatuses have been proved in different species, including humans. Among the different systems that compose the living beings, the central nervous system (CNS) and the immune one are recognized as ECS-dependent for its pivotal regulative role. Considering the CNS more in detail, eCBs, the related receptors, and metabolic enzymes are expressed throughout the whole HPG axis, suggesting a hypothetical, nowadays proved, interaction with the gonadal hormone signaling network in both males and females. This interplay, which is translated in an active regulation of GnRH and the downstream FSH and LH control of sex steroids, leads to the conclusion that ECS is strongly involved in the multifactorial processes of reproductive functions.

The reproductive biology research field in the last decades is full of numerous evidences from a wide range of study models – including mouse, large mammals, and humans – that highlight the manifold roles of ECS in reproduction, both in physiological (e.g. gametogenesis, fertilization competence, pregnancy, and labor) and pathological (e.g. polycystic ovary syndrome, endometriosis, and gynecological cancers) conditions.

In males, AEA, 2-AG and their metabolic receptors have a pivotal role in the regulation of sperm functions necessary to acquire fertilization competence, in terms of sperm motility, capacitation, acrosome reaction, and mitochondrial activity during hyperactivation, through CB_1, CB_2, and TRPV1. In females, eCBs and eCB-like compounds are associated with optimal follicular and oocyte maturation, even if the mechanisms of action have not yet been fully explored. In this matter, it is important to consider the use of animal models, as mouse, to finely comprehend the basal physiology and regulation directed by the ECS in the main common processes. Among these, oocyte meiotic maturation is one of the most important and, at the same time, less investigated.
The aim of this thesis is to clarify the role of ECS during the different stages of oocyte meiotic maturation. By using the mouse as a model, the expression levels of the four main receptors: CB_1, CB_2, GPR55, and TRPV1 have been evaluated by assessing mRNA and

protein levels of these CBRs, together with their localization, in oocytes arrested at GV-, MI- and MII-stage. To clarify their role during meiotic maturation, experiments with receptor-specific antagonists have been carried out.

Chapter 5

Materials and methods

5.1. Experimental design

To fulfill the aim of the thesis, adult female mice underwent one cycle of complete ovarian stimulation (i.e. PMSG and hCG injections, 5 IU/0.1 mL, i.p.). Oocytes were retrieved at different stages of *in vivo* meiotic maturation (germinal vesicle, GV; metaphase I, MI; metaphase II, MII) and processed, as explained in the following sections, for the evaluation of i. CB_1, CB_2, GPR55, and TRPV1 mRNA and protein expression levels (by Real-time PCR and Western blot, respectively); ii. protein localization (by immunofluorescence analysis under confocal microscopy).

Since TRPV1 was almost undetectable in oocytes of all stages, the roles of CB_1, CB_2, and GPR55 during the processes of meiotic resumption and maturation were more accurately investigated by using receptor-specific antagonists (SR141716A, SR144528, and ML193 trifluoroacetate, respectively). Thus, intraoocyte cAMP concentration was detected in oocytes undergoing GVBD, while the percentage of MI and MII oocytes with normal spindle morphology was assessed by confocal microscopy.

5.2. Chemicals

Hepes-buffered Eagle's minimal essential medium (MEM-HEPES) and MEM-α modification (αMEM), penicillin, and streptomycin were purchased from ThermoFisher Scientific (MA, USA). Pregnant mare serum gonadotropin (PMSG, Folligon) and human chorionic gonadotropin (hCG, Corulon) were obtained from Intervet International B. V. (AN Boxmeer, Nederland). SR141716A (SR1; cat. 0923/10) and SR144528 (SR2; cat. 5039/10) were obtained from Tocris Bioscience, Bristol, UK). ML193 trifluoroacetate (ML193, cat. SML1340) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies against rabbit CB_1 (cat. 101500), CB_2 (cat. 101550), GPR55 (cat. 10224), and specific blocking peptides for CB_1 (cat. 301500), CB_2 (cat. 301550) and GPR55 (cat. 10225) antibodies were obtained from Cayman Chemical (Anne Arbore, MI, USA). Rabbit TRPV1 (cat. TA336871) antibody was obtained from OriGene

Technologies, Inc. (Rockville, MD, USA). Cyanine 3 bisacid (Cy-3) anti-rabbit (cat. A10520) and Alexa Fluor 488 anti-mouse (cat. A32723) used as secondary antibodies for immunofluorescence analysis, and goat anti-rabbit horseradish peroxidase (HRP, cat. 111-035-003), used as secondary antibody for western blotting, were obtained from ThermoFisher Scientific. All the other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), and were of the purest analytical grade.

5.3. Animals & Ethical approval

Mus musculus Swiss CD1 female mice (23–25 days old; Charles River Laboratories, Lecco, Italy) were housed in an animal facility under controlled temperature (21±1 °C) and light (12 h light/day) conditions, with free access to food and water.

Five IU of PMSG were injected intraperitoneally (i.p.) in all mice. Forty-two to forty-four hours later, mice were in part sacrificed to obtain preovulatory germinal vesicle (GV)-stage oocytes, in part underwent i.p. injection of hCG (5 IU). After 3–12 h (depending on experimental protocols), mice were sacrificed to obtain oocytes at different *in vivo* maturation stages. Specifically, MI and MII oocytes were retrieved after 8 and 12 h of hCG injection, respectively.

All experimental procedures involving animals and their care were performed in conformity with national and international laws and policies (European Economic Community Council Directive 86/609, OJ 358, 1, 12 December, 1987; European Parliament Council Directive 2010/63/EU, OJ L 276, 20 October 2010; Italian Legislative Decree 116/92, Gazzetta Ufficiale della Repubblica Italiana n. 40, 18, February 1992; National Institutes of Health Guide for the Care and Use of Laboratory Animals, NIH publication no. 85-23, 1985). The project was approved by the Italian Ministry of Health and by the Institutional Review Board of the University of L'Aquila. The method of euthanasia consisted of an inhalant overdose of carbon dioxide (CO_2, 10–30%), followed by cervical dislocation. All efforts were made to minimize suffering. A total number of 200 mice were utilized to perform all the experimental procedures.

5.4. Collection of oocytes at different stages of *in vivo* meiotic maturation

Fully-grown, GV-stage oocytes surrounded by cumulus cells (oocyte-cumulus cell complexes, OCCs) were collected in MEM-HEPES supplemented with 0.23 mM pyruvic acid, 2 mM l-glutamine and 0.3% BSA (here referred as MEM), and immediately devoid of cumulus cells by gentle pipetting. MI and MII oocytes were recovered from ovaries 8 h after hCG and from fallopian tubes 12 h after hCG, respectively. When needed, cumulus cells were removed by a brief hyaluronidase treatment. Oocytes were either stored at -80°C or immediately utilized for morphological or molecular analysis.

5.5. mRNA expression: quantitative Real-Time PCR analysis

Total RNA was extracted from GV, MI and MII oocytes (20 oocytes/sample) using the RNeasy extraction kit (Qiagen, Crawley, UK), following the manufacturer instructions. Starting with 100 ng of RNA, complementary DNA (cDNA) was prepared using M–MLV reverse transcriptase kit (ThermoFisher Scientific, Waltham, MA, USA). Quantitative PCR analysis was performed using SYBR Green I Master and the LightCycler 480 System (Roche, Basel, Switzerland) on a DNA Engine Opticon 2 Continuous Fluorescence Detection System (BioRad, Hercules, CA, USA). The reaction was performed using the following qRT-PCR program: 95 °C for 10 min, followed by 40 amplification cycles of 95 °C for 10 s, 57 °C for 30 s, and 72 °C for 30 s. The primer used for the amplification of *Cnr1*, *Cnr2*, *Gpr55*, and *Trpv1* (Compagnucci *et al.*, 2013; Pucci and D'Addario, 2016) are listed in Table 4 and all the data were normalized to the endogenous reference gene β-Actin. Relative quantitation of mRNAs was performed by the comparative ΔΔCt method (Pucci *et al.*, 2012).

Gene	Corresponding Protein	PCR Primers	Annealing T (°C)	Reference
Cnr1	CB_1	Fw: 5′-CCAAGAAAAGATGACGGCAG-3′ Rev: 5′-AGGATGACACATAGCACCAG-3′	57	Compagnucci *et al.*, 2013
Cnr2	CB_2	Fw: 5′-TCGCTTACATCCTTCAGACAG-3′ Rev: 5′-TCTTCCCTCCCAACTCCTTC-3′	57	Compagnucci *et al.*, 2013
Gpr55	GPR55	Fw: 5′-ATTCGATTCCGTGGATAAGC-3′ Rev: 5′-ATGCTGATGAAGTAGAGGC-3′	57	Pucci and D'Addario, 2016
Trpv1	TRPV1	Fw: 5′-TGAACTGGACTACCTGGAAC-3′ Rev: 5′-TCCTTGAAGACCTCAGCATC-3′	57	Pucci and D'Addario, 2016
Actb	Actin	Fw: 5′-CTGTCGAGTCGCGTCCACCC-3′ Rev: 5′-GCTTTGCACATGCCGGAGCC-3′	57	Compagnucci *et al.*, 2013

Table 4. List of the primers utilized for quantitative real-time PCR analysis.

5.6. Protein expression: Western Blotting analysis

GV, MI, and MII oocytes (150 oocytes/sample) were lysed in sample buffer containing protease inhibitors (2 mM phenylmethyl sulphonyl fluoride, 10 μg/mL aprotinin, 0.1 mM sodium pyrophosphate, 10 mM sodium fluoride, and 1 mM sodium orthovanadate). Lysates were separated by electrophoresis and transferred to nitrocellulose membranes (Hybond C Extra, Amersham, UK). Membranes were incubated overnight at 4 °C with antibodies anti-CB_1 (1:200), -CB_2 (1:200), -GPR55 (1:200), and -TRPV1 (1:200). HRP-conjugated goat anti-rabbit IgG (1:5000) was used as secondary antibody (1 h, room temperature: r.t.); the peroxidase activity was detected using a SuperSignal West Pico Chemiluminescent substrate. Membranes were examined by Alliance LD2-77WL imaging system (Uvitec, Cambridge, UK). Densitometric quantification was performed with the public-domain software NIH Image 167 V.1.62 and standardized using tubulin as loading control. Negative controls (NC) were prepared using specific blocking peptides (for CB_1, CB_2, and GPR55). For the anti-TRPV1 antibody used in this study, there are no blocking peptides commercially available.

5.7. Protein localization: Immunofluorescence analysis

To detect the presence and distribution of receptors at GV, MI and MII stage, oocytes (15/sample) were fixed in 4% paraformaldehyde for 10 min at r.t., permeabilized with 0.1% Triton X-100 for 30 min at 37 °C (Rossi *et al.*, 2006). Afterward, oocytes were

incubated with the following primary antibodies diluted in a PBS blocking solution containing 2% BSA, 2% powder milk, 2% normal goat serum, 0.1 M Glycine, and 0.01% Triton X-100: CB_1 (1:100), CB_2 (1:100), GPR55 (1:200) and TRPV1 (1:200) for 1 h at 37 °C.

To detect the presence of receptors at the oocyte plasma membrane (oolemma), oocytes were firstly incubated in Tyrode's solution at pH 2.5 to remove zona pellucida (zona-free, ZF), and then fixed in 4% paraformaldehyde (0.2% BSA in 0.1 M PBS, pH 7.4). Afterward, oocytes were incubated for 1 h at 37 °C with the following primary antibodies diluted in 0.2% BSA-PBS: CB_1 (1:100), CB_2 (1:100), GPR55 (1:200) and TRPV1 (1:200). Oocytes were then incubated with Cy-3 anti-rabbit secondary antibody (1:200) for 1 h at 37 °C, mounted using 1.5 µl of 50% glycerol/PBS solution containing sodium azide and DAPI (1:1000) to label nuclei (Di Nisio *et al.*, 2018).

To monitor the distribution of CB_1 in the oolemma during the transition from GV to MI, ZF-oocytes were collected at different timings after hCG injection (0, 3, 5, 8 h; 15 oocytes/time point), and incubated with anti-CB_1 antibody (1:100)-Cy-3 anti-rabbit secondary antibody (1:200) for 1 h at 37 °C each.

For each set of experiments, NCs were prepared using specific blocking peptide (for CB_1, CB_2, and GPR55) and omitting the primary antibody for TRPV1 before the addition of the secondary antibody. All the oocytes were observed by confocal microscopy (Leica System TCS SP5 confocal microscope, Wetzlar, Germany). Images were taken at the equatorial plan using the LAS AF software (Leica Microsystems).

For image analysis, data from high-resolution images of 6 oocytes from 3 independent experiments were acquired for each sample. Quantification of the CBRs' intracellular mean fluorescence was carried out using the public-domain software NIH Image 167 V.1.62 after the subtraction of the background intensity calculated from the NCs' images.

5.8. Effects of CBRs antagonists on oocyte *in vitro* meiotic maturation processes

5.8.1. Kinetics of oocyte germinal vesicle breakdown and intraoocyte cAMP content

OCCs were collected in MEM supplemented with cilostamide (1 µM) to maintain meiotic arrest (Coticchio *et al.*, 2004). After washing, OCCs were (i) in part devoid of somatic cells to obtain GV stage oocytes (t = 0) that were immediately stored at -80 °C;

(ii) in part cultured at 37 °C in 5% CO_2 for 30, 60, 90, and 120 min in the absence (control, Ctr) or presence of CB_1-specific antagonist SR1 and CB_2-specific antagonist SR2, used alone or in combination. The antagonists were all used at 0.5 μM, which was the lowest effective dose calculated after preliminary experiments, in line with a previous study (Pucci *et al.*, 2012). The culture medium utilized was αMEM supplemented with 0.23 mM pyruvate, 2 mM l-glutamine and 0.05% DMSO (hereafter referred to as αMEM-DMSO). At each time point, OCCs were deprived of cumulus cells to record the percentage of GVs, according to the presence or absence (GVBD) of the GV in the ooplasm.

The amount of cAMP was determined in groups of 120 oocytes incubated stored after GV assessment, by using a Cyclic AMP EIA Kit (581001, Cayman Chemical Company, Anne Arbore, MI, USA) according to manufacturer's instructions. Absorbance at 420 nm was measured in a Model 550 microplate reader (BioRad).

5.8.2. Polar body I emission and spindle formation

To evaluate the effects of antagonists on the morphology of MI spindle, OCCs were cultured for 8 h in 300 μL αMEM-DMSO in the absence (Ctr, n = 30 oocytes) or presence of 0.5 μM SR1 (n = 45 oocytes), SR2 (n = 45 oocytes), ML193 (n = 80 oocytes), or a combination of the three antagonists (SR1 + SR2 + ML193; n = 50 oocytes). Following cumulus cells removal, only oocytes undergoing GVBD were fixed as described in the following procedure.

The analysis of antagonists' effect on PBI and MII spindles were performed by retrieving OCCs 8h after hCG, i.e. at MI *in vivo* maturation stage, and then by culturing them in the absence (Ctr, n = 30 oocytes) or presence of 0.5 μM SR1 (n = 30 oocytes), SR2 (n = 50 oocytes) or ML193 (n = 80 oocytes), or a combination of the three antagonists (SR1+SR2+ML193; n = 50 oocytes) for 5 h. This experimental design was chosen in order to reduce the times of oocyte *in vitro* culture. By the end of the culture period, the percentage of normal PBI was recorded, and oocytes were then fixed as described above. Afterward, oocytes were incubated for 1 h at 37 °C with anti-tubulin primary antibody (1:100) and then with anti-mouse secondary antibody conjugated with Alexa Fluor 488 (1:1000). Chromosomes were labeled with DAPI (1:1000) (Rossi et al., 2006; Di Nisio et al., 2018). Spindle sizes, i.e. length (Wang *et al.*, 2016) and area (Sanfins *et al.*, 2003),

were measured by the software ZEN 2009 Light Edition (Carl Zeiss MicroImaging GmbH), as described by Wang and collaborators (Wang *et al.*, 2016).

5.9. Statistical analysis

The experiments were replicated at least 3 times, and the obtained data were expressed as the mean ± S.E.M. Analyses for statistical significance were performed using:

- ANOVA followed by the Tukey-Kramer post-test for comparison of multiple groups,
- ANOVA followed by Bonferroni post-test for comparison among treatments and control groups and
- ANOVA followed by the chi-square test for comparison of percentages.

Results were considered significantly different when $P<0.05$.

Chapter 6

Results and role of the chance

6.1. CBRs mRNA expression during different stages of *in vivo* oocyte meiotic maturation

The Real-time PCR analysis of CBRs mRNA expression, reported in Figure 8, evidenced that *Cnr1*, *Cnr2*, and *Gpr55* levels dramatically dropped after GV during *in vivo* meiotic maturation (MI and MII *vs* GV, $P<0.05$). Differently, *Trpv1* mRNA expression level remained always very low throughout *in vivo* meiotic maturation (GV *vs* MI *vs* MII, $P>0.05$).

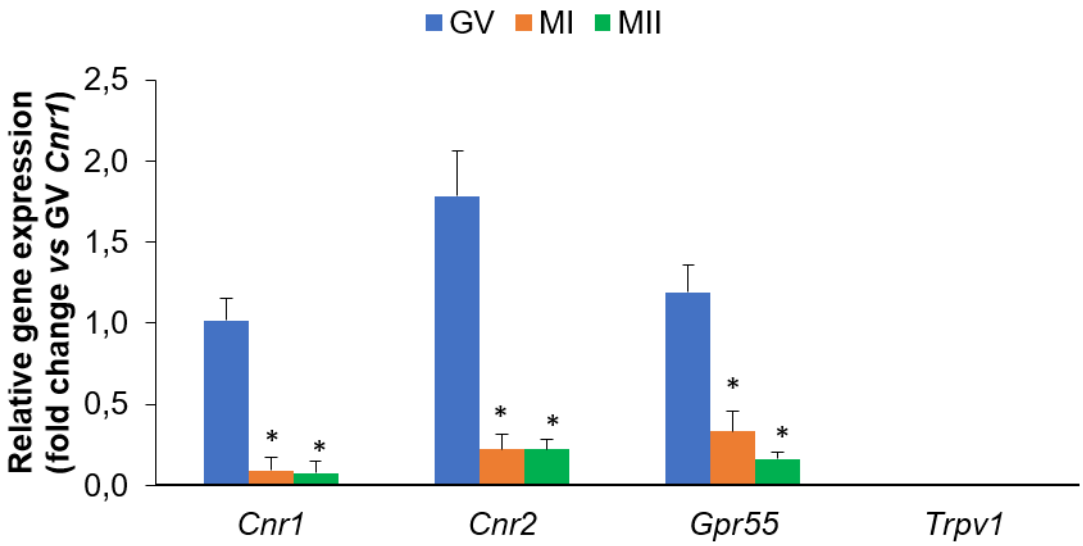


Figure 8. Expression levels of CBRs mRNA during mouse oocyte *in vivo* meiotic maturation. Real-time PCR of *Cnr1*, *Cnr2*, *Gpr55*, and *Trpv1*. Data were reported as $2^{-\Delta\Delta Ct}$ values calculated by Delta–Delta Ct ($\Delta\Delta$Ct) method *vs* germinal vesicle (GV) (*Cnr1*) group posed equal to 1. Expression was normalized to the housekeeping gene *Actb* and values were reported as mean ± SEM of 4 independent replicates. **P*<0.05 *vs* GV oocyte of the same experimental group.

6.2. CBRs protein expression during different stages of *in vivo* oocyte meiotic maturation

The analysis by Western blot of CBRs protein expression (Figure 9) in lysates of *in vivo* matured oocytes indicated that, at GV stage, CB_1, CB_2, and GPR55 were equally expressed. At MI stage, CB_1 protein level decreased significantly (GV *vs* MI and MII, $P<0.05$), whereas CB_2 and GPR55 showed a rapid increase (GV *vs* MI: $P<0.05$). At MII,

GPR55 contents were exponentially rising in comparison with both GV and MI (GV *vs* MI *vs* MII, $P<0.05$). Concerning TRPV1, its signal was barely detectable at any meiotic stage ($P>0.05$).

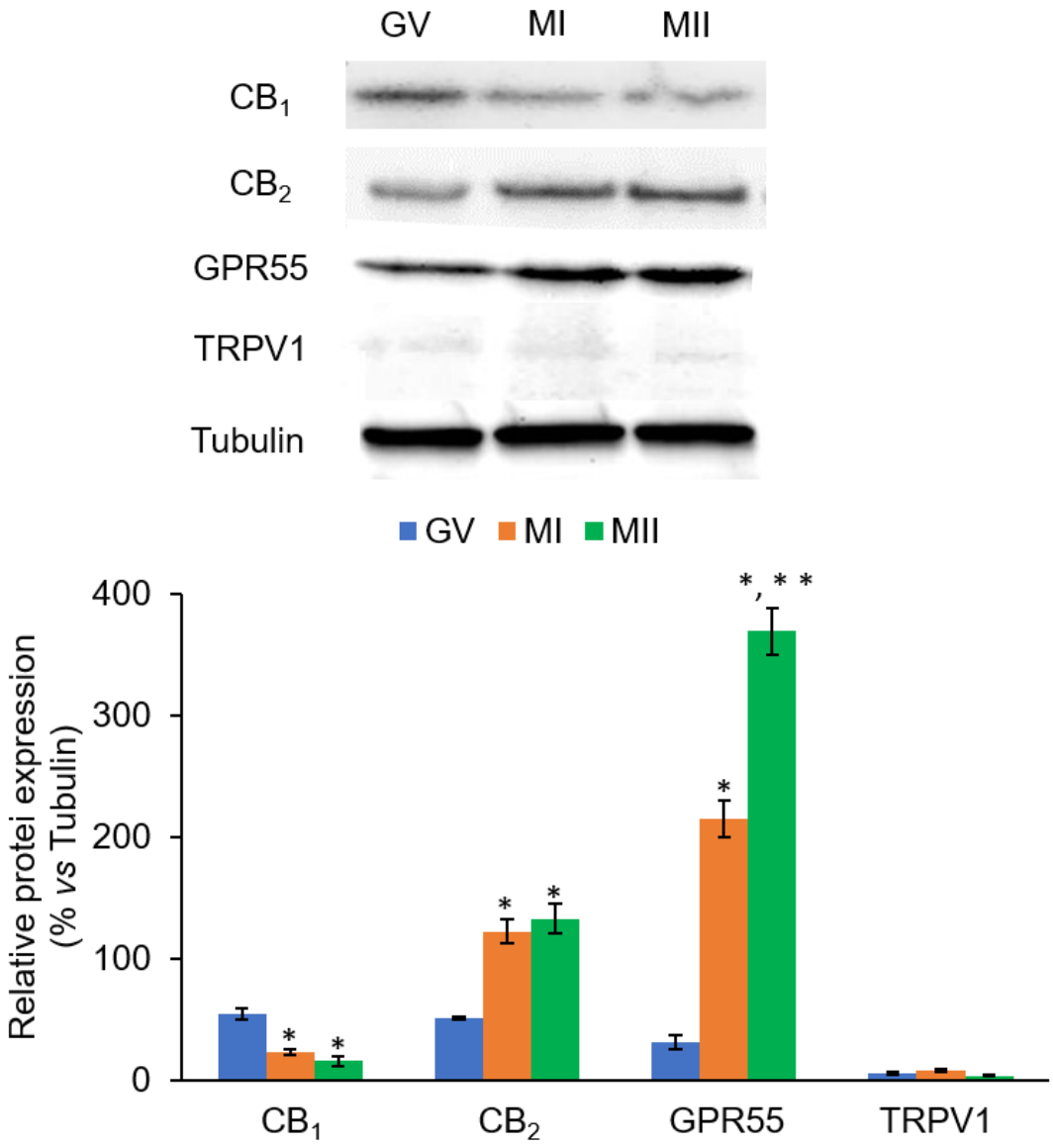


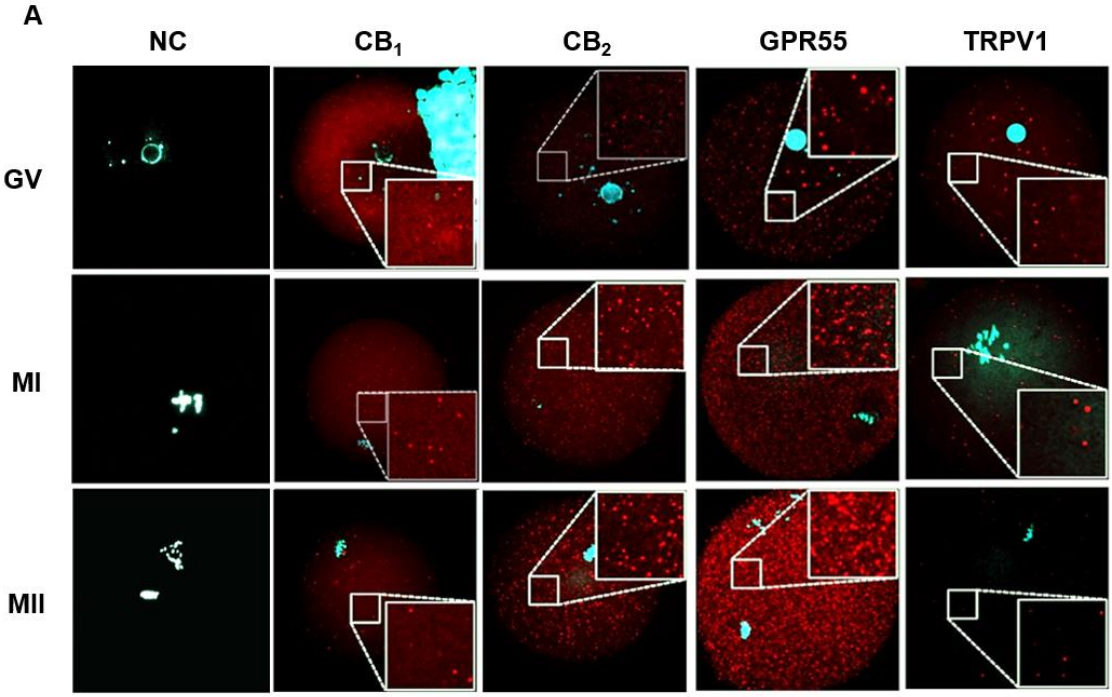
Figure 9. Expression levels of CBRs protein during mouse oocyte *in vivo* meiotic maturation. group. Representative western blot and quantification of CB_1, CB_2, GPR55, and TRPV1 protein contents. Data are expressed as mean ± SEM of each receptor content after normalization with the housekeeping α/β-tubulin, used as loading control. Experiments were repeated 3 times. **P*<0.05 *vs* GV oocyte of the same experimental group; ***P*<0.05 *vs* MI of the same experimental group. GV = germinal vesicle; MI = metaphase I; MII = metaphase II.

6.3. CBRs protein localization during different stages of *in vivo* oocyte meiotic maturation

At GV stage, the cytoplasmic CBRs immunolocalization performed by confocal microscopy presented a homogeneous distribution of all receptors (Figure 10). In

particular, CB_1's immunostaining appeared more intense than CB_2's and GPR55's. In MI oocytes, CB_1 fluorescence intensity was restrained in fewer dots, while CB_2 and GPR55 cytoplasmic signal increased, in accordance with Western blot results. At MII stage, CB_1 fluorescence decreased even more in comparison with GV and MI, whilst CB_2 signal remained unchanged and GPR55 immunostaining was remarkably enhanced, as reported in the fluorescence values table in Figure 10. In keeping with molecular data from Western blot analysis and Real-time PCR, TRPV1 fluorescent signal was faintly detectable throughout all the stages of meiotic maturation.

A

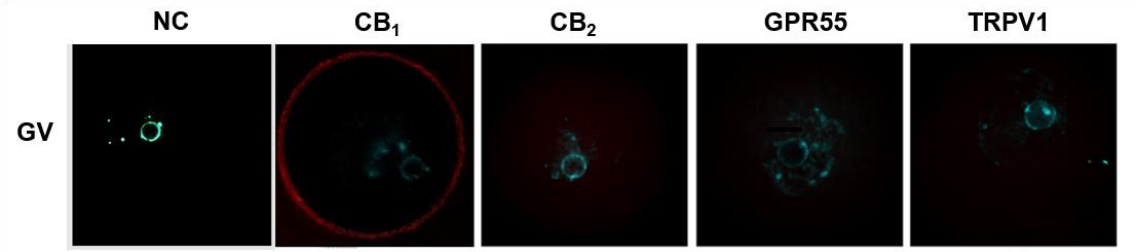


B

Oocyte stage	Fluorescence (AU)			
	CB_1	CB_2	GPR55	TRPV1
GV	546 ± 5	185 ± 7	204 ± 8	188 ± 10
MI	389 ± 16*	417 ± 19*	467 ± 18*	175 ± 14
MII	356 ± 15*	460 ± 9*	926 ± 20*,**	133 ± 30

Figure 10. (**A**) Localization of CB_1, CB_2, GPR55, and TRPV1 receptors in mouse oocytes collected at various stages of *in vivo* meiotic maturation. Receptors were labeled with Cy-3 (red), DNA was counterstained by DAPI (cyan). In the upper right-hand corner of GV CB_1, the strong DAPI staining is due to undetached cumulus cell nuclei. Each image was taken at the equatorial plane of the oocyte. Magnification: ×630. Each inset represents a magnified part of ooplasm. GV = germinal vesicle; MI = metaphase I; MII = metaphase II; NC = negative control. (**B**) Mean fluorescence of CB_1, CB_2, GPR55, and TRPV1 receptors in mouse oocytes collected at different stages of meiotic maturation. Values are expressed as arbitrary units (AU) and are reported as mean ± SEM of 6 oocytes from 3 independent experiments. **P*<0.05 *vs* GV oocyte of the same experimental group; ** *P*<0.05 *vs* MI of the same experimental group.

The analysis of CBRs localization at the oolemma (the plasma membrane of oocytes) evidenced that only CB_1 was present at GV stage, while no signal was detected for any CBRs analyzed at both MI and MII stages (Figure 11).



Oocyte stage	Presence (+)/Absence (-) in oolemma			
	CB_1	CB_2	GPR55	TRPV1
GV	+	-	-	-
MI	-	-	-	-
MII	-	-	-	-

Figure 11. Distribution of CB_1, CB_2, GPR55, and TRPV1 in the oolemma. Only GV oocytes showed detectable signals. Qualitative data are reported in the table and are expressed as presence (+) or absence (-) of fluorescence in 15 oocytes/sample. Each image was taken at the equatorial plane of the oocyte. Cy-3: red, DAPI: cyan. Magnification: ×630.

In order to define CB_1 disappearance dynamics during the GV-MI transition, oocytes were collected at different timings after hCG injection: 0h, 3h, 5h and 8h (Figure 12). The homogenous distribution of CB_1 recorded at time 0 became restricted to small microdomains (likely related to the presence of lipid rafts) after 3h. After 5h, CB_1 was more and more compartmentalized in microdomains, and finally, it completely disappeared after 8h, whereupon oocytes reached the next stage of meiotic maturation (MI).

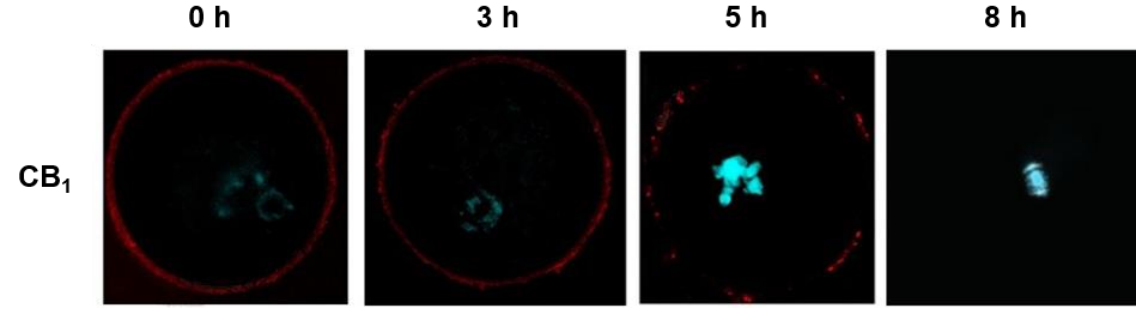


Figure 12. CB_1 localization at oolemma during in vivo GV-MI transition. CB_1 distribution, analyzed at different times after human chorionic gonadotropin (hCG), changed from uniformly homogeneous (0–3 h) to dotted clusters (5 h), until complete disappearance at MI (8 h). Each image was taken at the equatorial plane of the oocyte. Cy-3: red, DAPI: cyan. Magnification: ×630.

6.4. Effects of CBRs antagonists on intraoocyte cAMP concentration

The effects of CB_1- and CB_2-selective antagonists (SR1 and SR2, respectively) on the kinetics of meiotic resumption were investigated by using antagonists (alone or in combination) at the concentration of 0.5 μM. As reported in Figure 13, after 30 min of culture nearly all oocytes stayed arrested at GV stage (Ctr *vs* SR1, SR2, and SR1+SR2; $P>0.05$). After 60 min, GVBD occurred in about 40% of Ctr oocytes, while only 20% of antagonists treated oocytes (alone or in combination) underwent GVBD (Ctr *vs* SR1, SR2, and SR1+SR2; $P<0.05$). Ninety min after the beginning of the culture, about 95% of Ctr oocytes underwent GVBD in contrast with the 56% of treated oocytes (SR1, SR2, SR1+SR2) (Ctr *vs* SR1, SR2, and SR1+SR2; $P<0.05$). At 120 min, all oocytes had resumed meiosis, despite the treatment (Ctr *vs* SR1, SR2, and SR1+SR2; $P>0.05$). At any timing of the kinetics, the antagonists used alone or in combination presented similar percentages of oocytes resuming meiosis (SR1 *vs* SR2 *vs* SR1+SR2; $P>0.05$).

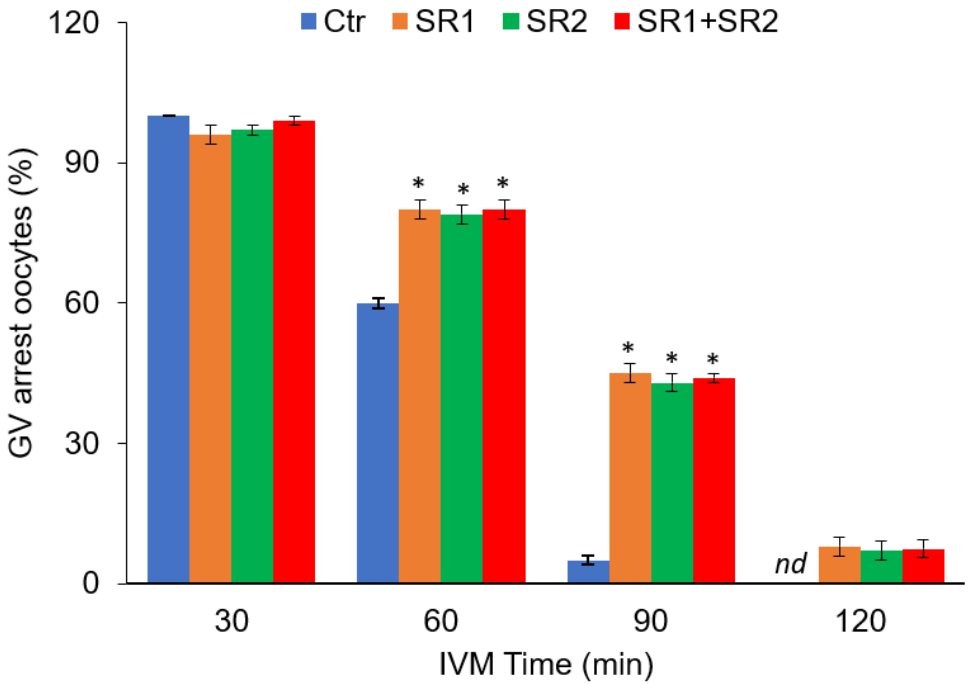


Figure 13. Effects of CBRs antagonists on the kinetics of oocyte germinal vesicle breakdown (GVBD). Oocyte–cumulus cell complexes (OCCs) were matured *in vitro* in the presence of 0.05% DMSO (Ctr) or SR1, SR2, and SR1 + SR2 (0.5 μM) for 30, 60, 90, and 120 min. Data are expressed as percentage of GV arrested/total oocytes. *nd*: not detectable.

On the basis of the above-mentioned results, in the next set of experiments, the hypothesis of CB_1- and CB_2-dependent activation of $G_{\alpha i}$ proteins (Oddi *et al.*, 2018) has been tested to investigate their possible involvement in the meiotic resumption through the modulation of cAMP intraoocyte concentration (Horner *et al.*, 2003; Vaccari *et al.*,

2008). For that purpose, cAMP concentration was assessed in *in vitro* cultured oocytes up to 120 min in the absence (Ctr) or presence of SR1, SR2, and SR1+SR2 (Figure 14). Initially, the detected cAMP concentration was 0.30 ± 0.01 fmol/oocyte, and after 30 min it slightly decreased equally in all the experimental conditions (Ctr *vs* SR1, SR2, and SR1+SR2; $P>0.05$). After 60 min, the cyclic nucleotide amount sharply decreased in Ctr (0.16 ± 0.007 fmol/oocyte) and, at a lesser extent, in SR1-, SR2-, and SR1+SR2-treated cells (~0.21 ± 0.012 fmol/oocyte) (Ctr *vs* SR1, SR2, and SR1+SR2; $P<0.05$). At 90 min, while in Ctr oocytes cAMP concentration was undetectable, in antagonists-treated cells was ~0.11 ± 0.015 fmol/oocyte (Ctr *vs* SR1, SR2, and SR1+SR2; $P<0.05$). At the last timing (120 min), cAMP was undetectable in all experimental groups (Ctr *vs* SR1, SR2, and SR1+SR2; $P>0.05$). As for the GVBD kinetics, similar results were obtained in antagonists treatment groups, despite if used alone or in combination (SR1 *vs* SR2 *vs* SR1+SR2; $P>0.05$).

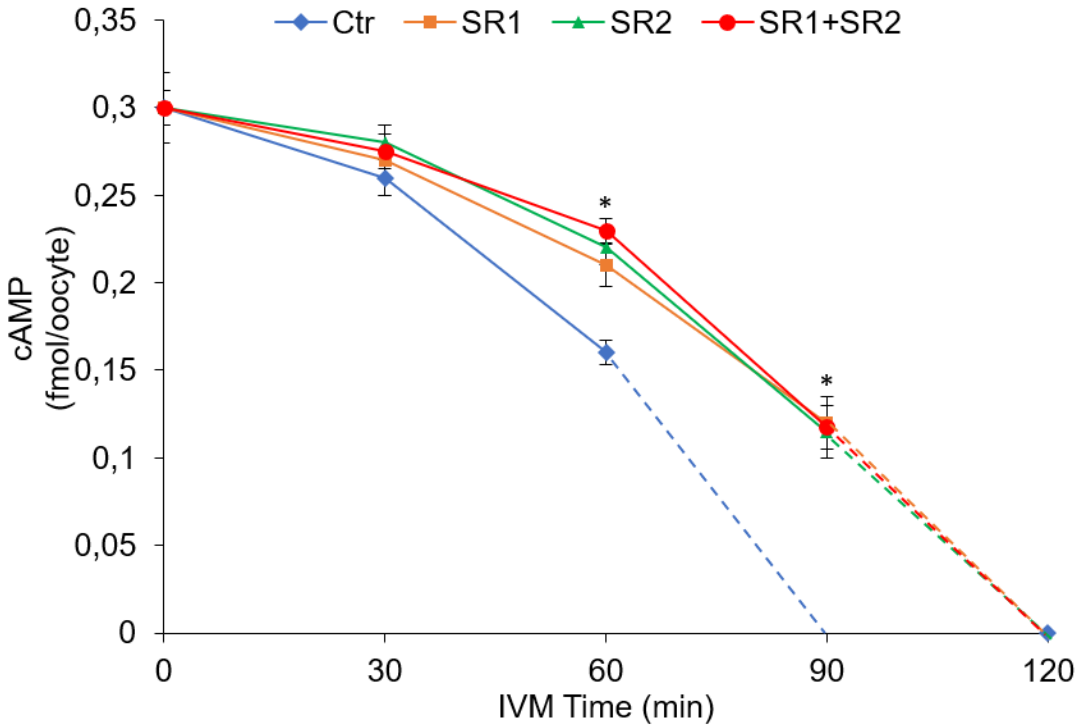


Figure 14. Effects of CBRs antagonists on cAMP concentration (fmol/oocyte). cAMP values were assayed in oocytes cultured *in vitro* for 30, 60, 90, and 120 min as Ctr or in the presence of SR1, SR2, and SR1 + SR2 (0.5 μM). Data are expressed as mean ± SEM of 4 independent experiments. *$P<0.05$ *vs* Ctr oocytes of the same experimental group. *nd*: values below the limit of detection of the assay (0.1 pmol/mL).

6.5. Effects of CBRs antagonists on polar body I emission and spindle formation

The evaluation of CBRs role in oocyte meiotic maturation was then focused on the putative role of CB_1, CB_2 and/or GPR55 antagonists (SR1, SR2, and ML193, respectively) on the extrusion of PBI and/or spindle morphology of both MI- and MII-stages. Generally, the presence of CBRs antagonists (all used at 0.5 μM) did not affect the IVM process (Table 5). In fact, the percentages of MI- (>90%; Ctr *vs* antagonists, $P>0.05$) and MII-stage oocytes (~80% PBI; Ctr *vs* antagonists, $P>0.05$) were similar among the experimental groups. In the presence of a mix of the three antagonists, comparable results were obtained (MI: >90%; MII: ~82% PBI; Ctr *vs* SR1+SR2+ML193; $P>0.05$).

Treatment	Oocytes (N)	MI (%)	MII (%)
Ctr	30	96.02 ± 3.98	81.25 ± 2.13
SR1	45	95.80 ± 3.15	82.93 ± 1.42
SR2	45	94.56 ± 5.03	80.95 ± 3.26
ML193	80	95.51 ± 4.36	81.16 ± 2.70
SR1+SR2+ML193	50	94.83 ± 2.91	81.60 ± 2.24

Table 5. Percentages of oocytes at MI- or MII-stage after *in vitro* maturation (IVM) in the presence or absence (Ctr) of CBRs antagonists. All oocytes were analyzed by confocal microscopy for the evaluation of spindle morphology.

Regarding spindle morphology, the presence of SR1 or SR2 did not alter chromosome alignment at metaphase plate of both maturation stages (Figure 15; >95%; Ctr *vs* SR1 and SR2; $P>0.05$) nor spindle morphology (Figure 15) or size (Table 6), since >80% of SR1- and SR2-treated oocytes showed a mean spindle length (MI: ~35.25 ± 0.19 μm; MII: ~32.39 ± 0.24 μm) and area (MI: ~569.78 ± 3.17 μm^2; MII: ~481.52 ± 1.64 μm^2) similar to Ctr ($P>0.05$). On the contrary, ML193-treated oocytes showed a dramatically negative effect of this antagonist on overall spindle morphology at both MI- and MII-stage (Figure 15). As a matter of fact, ~75% of oocytes at MI and MII stages cultured in the presence of ML193 displayed significantly shorter (MI: 24.34 ± 0.44 μm; MII: 22.96 ± 0.30 μm) and with smaller areas (MI: 376.44 ± 2.42 μm^2; MII: 247.60 ± 1.31 μm^2) spindles

compared to Ctr (P<0.05). Despite the effect on spindle morphology and size, ML193-treated oocytes showed a normal chromosome alignment on both MI and MII metaphase plates (Figure 15).

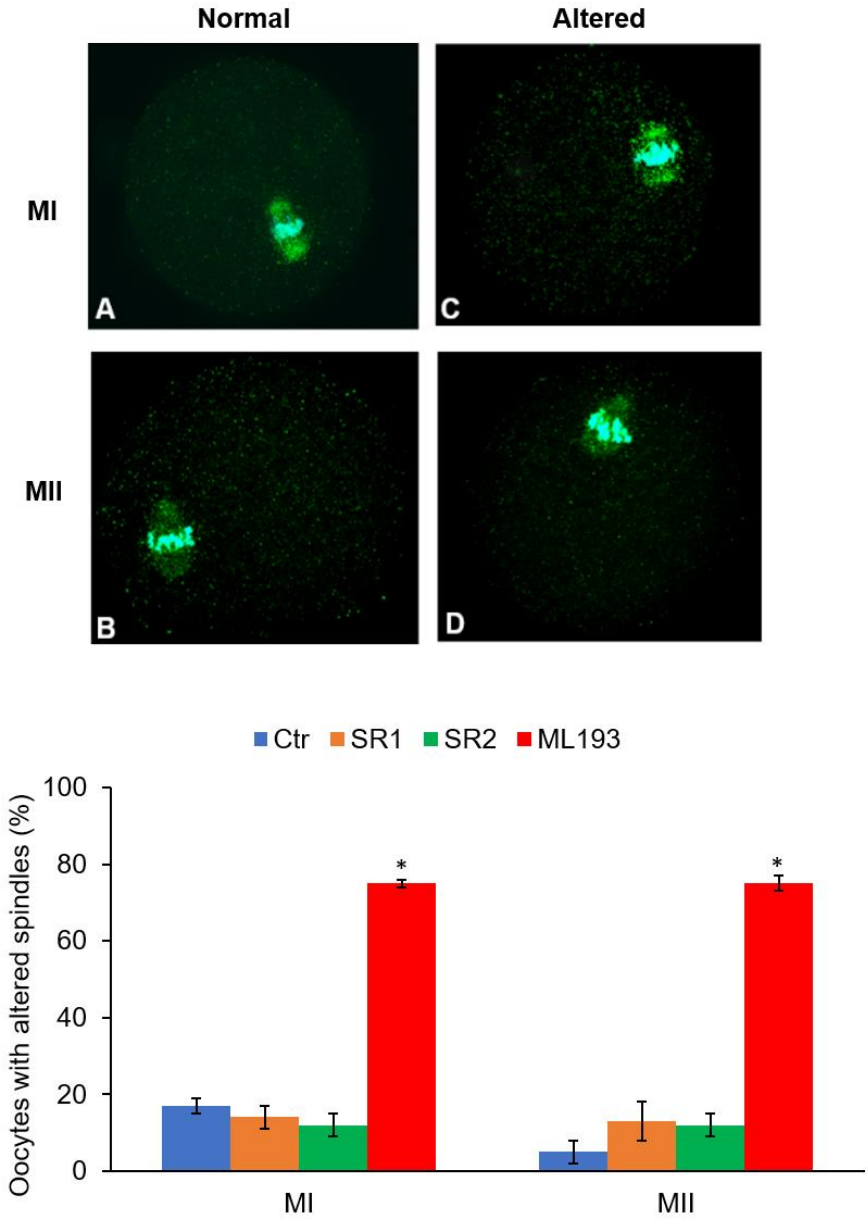


Figure 15. MI and MII spindle morphology. To obtain MI oocytes, OCCs were cultured in presence or absence (Ctr) of SR1, SR2, and ML193 (0.5 μM) for 8 h. To obtain MII oocytes, OCCs were collected 8 h after hCG and cultured *in vitro* for 5 h. Spindles were stained with anti-α/β-tubulin antibody (green) and chromosomes with DAPI (cyan). Representative images of normal MI (**A**) and MII (**B**) spindles and of altered MI (**C**) and MII (**D**) spindles. Magnification: ×630. In the graph are reported oocytes percentages of MI and MII altered spindles in the presence of CBRs antagonists. Data are expressed as mean ± SEM of 3 independent experiments. *P<0.05 *vs* Ctr oocytes of the same experimental group.

Treatment	MI			MII		
	N°	Length (µm)	Area (µm^2)	N°	Length (µm)	Area (µm^2)
Ctr	30	35,20±0,13	574,83±1,51	30	32,64±0,17	482,58±1,32
SR1 (0.5 µM)	45	34,58±0,31	563,81±3,38	30	32,58±0,21	480,36±2,01
SR2 (0.5 µM)	45	35,96±0,14	570,69±4,61	50	31,96±0,34	481,63±1,59
ML193 (0.5 µM)	80	24,34±0,44*	376,44±2,42*	80	22,96±0,30*	247,60±1,31*

Table 6. Spindle measurements (length and area) in the presence or absence (Ctr) of CBRs antagonists, all used at 0.5 µM. are expressed as and areas as. N° = number of oocytes analyzed per group. Values are expressed as arbitrary units µm (length) and µm^2 (area) and are reported as mean ± SEM. *P<0.05 *vs* Ctr oocyte of the same experimental group.

Chapter 7

Discussion and conclusions

The influence of the ECS in several fields of female reproduction in physiological and pathological conditions has been a central debate during the last decades. Considering its highly conserved expression in different species of animals, especially mammals, this system is considered as a key "gear" in the wide machinery of reproduction. In fact, thanks to studies on genetically modified animals, in particular mice, it has been proved that a fine physiological succession of events leading to the final successful pregnancy outcome is smartly connected to a balanced tune of endocannabinoids during the various steps of this process (Wang *et al.*, 2006c; Wang *et al.*, 2008; Sun *et al.*, 2010). Additionally, mouse models KO for genes encoding enzymes or receptors of ECS proved to be infertile, thus filling one more piece in the puzzle of endocannabinoid-dependent regulation of reproductive processes, even though it is possible that other signaling pathways could compensate the loss of ECS machinery (El-Brolosy and Stainier, 2017). Despite the large amount of data present in literature about the role of ECS in processes from embryo implantation onward, it is still not clear how this system could interact with the regulation of ovarian function, especially in oocyte meiotic maturation.

In this context, this thesis provided a detailed characterization of the panel of the main CBRs during the three stages of oocyte meiotic maturation (GV, MI, MII), demonstrating that three out of four eCB-binding receptors (namely CB_1, CB_2, and GPR55) are present in mouse oocytes, even if their expression undergoes modifications throughout *in vivo* oocyte maturation. Surprisingly, TRPV1 expression, at both mRNA and protein levels, was always low/undetectable, despite the maturation stage.

From the gene expression point of view, *Cnr1*, *Cnr2*, and *Gpr55* mRNAs are highly expressed in GV-stage oocytes. Instead, the dramatic decrease at MI- and MII-stage oocyte can be justified by the well-known transcription repression that occurs in oocytes following GVBD (Susor *et al.*, 2016). Our data regarding *Cnr2* expression are in contrast with the results obtained by López-Cardona and collaborators, who found this mRNA expressed at all stages of oocyte maturation (GV, MI, and MII) (López-Cardona *et al.*, 2017). The inconsistencies of these data could be related to the different methods utilized for the recruitment of MI- and MII-stage oocytes. In fact, while in our experiments MI-

stage oocytes are recruited directly from ovarian follicles after 8 h of hCG injection and mature oocytes (MII) from oviducts after 12 h of hCG injection, López-Cardona and coworkers collected them from the oviducts, 14 h after hCG injection (López-Cardona *et al.*, 2017). For what it concerns human oocytes, it seems that only *Cnr1* mRNA was found. However, PCR analysis was carried out on a mixture of oocytes at different stages of meiotic maturation, making impossible to discern eventual stage-specific modulations of mRNA expression (Peralta *et al.*, 2011).

Throughout the meiotic maturation process, CB_1 localization at GV oolemma changes during GVBD. In fact, this receptor appears to be gradually enclosed in clustered microdomains, which could be connected to the presence of lipid-rafts, phenomenon described under physiological (neuronal cells, CNS; Oddi *et al.*, 2012, Mangoura *et al.*, 2016) and pathological conditions (breast cancer cells, neuroblastoma cells; Sarnataro *et al.*, 2005, Hamtiaux *et al.*, 2011), and deeply connected to the activation status of CB_1 downstream signaling. The hypothetical reason for the CB_1 oolemma disappearance could reside in the necessity of shutting down this signaling to assure successful maturation and/or fertilization and/or early embryo development. Considering eCBs levels in the ampullary region of the oviduct, where fertilization occurs, AEA concentration is much lower in comparison with isthmus, in which CB_1-dependent acrosome reaction occurs in mammalian spermatozoa (Barboni *et al.*, 2011; Bernabò *et al.*, 2012). On the basis of these literature data, it can be assumed that the CB_1 receptor needs to be removed from oolemma in order to avoid abnormal signaling during fertilization. CB_1 and CB_2 are expressed during the early stages of embryogenesis, from 2 cell-stage onward and from zygote onward, respectively (Paria *et al.*, 1995; Yang *et al.*, 1996). Although, while CB_1 is clearly involved in embryo development (Paria and Dey, 2000; Oh *et al.*, 2013), the exact role of CB_2 remains still unclear, also because it appears unresponsive to any agonist stimulation (Paria and Dey, 2000; López-Cardona *et al.*, 2017).

CBRs protein analyses highlighted different receptor modulation during oocyte meiotic maturation. Indeed, in ooplasm TRPV1 is barely detectable at all stages of meiotic maturation, CB_1 decreases after GVBD, while CB_2 and GPR55 levels increase during the GV-MI transition, both reaching their maximum level at MII-stage. Results regarding CB_1 and CB_2 expression and localization are discordant with those presented by López-Cardona and collaborators (López-Cardona *et al.*, 2017). In fact, they found a different CB_1 distribution dependent on the maturation process (*in vivo* or *in vitro*): in GV *in vivo*

matured they found a predominant localization of this CBR in the periphery of the oocyte, while the GV oocytes collected for *in vitro* culture showed a homogeneous distribution in the cytoplasm. This differential localization at GV is not clear and fully justified since GV oocytes must have superimposable characteristics. López-Cardona and colleagues described a similar cytoplasmic CB_2 distribution during all the stages of meiotic maturation, whilst in the results here presented it is evident, both in confocal and Western blot data, an increase from GV to MI, after which this receptor expression is stabilized until MII. These differences could be due to the nonidentical procedure of oocyte retrieval, even though Western blot analysis of protein expression firmly reinforce confocal data here presented.

Literature data report the effect of CB_1 and CB_2 agonists on the kinetics of meiotic maturation. López-Cardona and collaborators evaluated the changes in the nuclear status of bovine oocytes matured *in vitro* in the presence of a CB1 agonist (HU210) and of a CB1/CB2 agonist (THC). Both compounds stimulated oocyte maturation during the first 2 hours, as demonstrated by the higher percentage of GVBD in comparison with control (López-Cardona *et al.*, 2016). A similar effect was described also for mouse oocytes incubated with THC (Totorikaguena *et al.*, 2019). These results mirror our findings regarding the delay of oocyte maturation occurring in the presence of CB1 and CB2 specific antagonists (SR1 and SR2, respectively). Therefore, a role of these cannabinoid receptors in the control of meiotic resumption can be hypothesized. Results here reported increase the knowledge of this important mechanism by linking the delay of meiotic resumption to the CBRs antagonist-dependent increase of intraoocyte cAMP concentrations.

Regarding TRPV1 and its almost undetectable presence in all the stages of oocyte maturation, the reasons could be that its pivotal role is restricted to spermatozoa, because together with CB_1 it regulates capacitation and their fertilizing ability (Francavilla *et al.*, 2009; Gervasi *et al.*, 2011, 2016). Alternatively, it is reported in literature the presence in mouse oocytes at different stages of maturation of another important member of the vanilloid family, TRPV3, which has a central role in the egg activation (Carvacho *et al.*, 2013; Lee *et al.*, 2016). Both the presence of eCBs-responsive TRPV3 (De Petrocellis *et al.*, 2012) in the oocyte and the well-known role of TRPV1 in sperm activation suggest that the expression of TRPV1 in oocytes is not indispensable and could create a state of unnecessary ligand competition disturbing the physiological fertilization processes.

Focusing on the roles of CBRs in oocyte maturation, the data here reported by using receptor-specific antagonists support a role for both CB_1 and CB_2 in controlling resumption of meiosis. By using SR1 and SR2 it is evident a significant delay in GVBD timings connected to a higher ooplasm concentration of cAMP. It is general knowledge that threshold levels of this specific cyclic nucleotide are indispensable for the fine regulation of oocyte meiotic resumption (Pan and Li, 2019). CB_1 and CB_2 are GPRs coupled to $G_{\alpha i}$ proteins, therefore modulating AC function, as already described in other cellular systems (Howlett and Abood, 2017; Oddi *et al.*, 2018). Considering oocyte meiotic arrest at GV stage, it is universally recognized the key role of the high intra-oocyte cAMP concentration (Horner *et al.*, 2003; DiLuigi *et al.*, 2008; Vaccari *et al.*, 2008), the production of which is guaranteed by GPR3 receptors activation of AC-type 3 through $G_{\alpha s}$ proteins coupling (Vaccari *et al.*, 2008; Conti, 2011). After gonadotropin stimulation during GVBD, the concentration of this cyclic nucleotide undergoes a sharp decrease, following gonadotropin-dependent closure of GCs-oocyte gap junctions and the activation of PDE3A (Conti, 2001; Gilchrist *et al.*, 2016; Richani and Gilchrist, 2018). Furthermore, in 2011, Lowther and collaborators described the mechanism of GPR3 endocytosis via a beta-arrestin/GRK-independent process and its relevance in oocyte meiotic resumption (Lowther *et al.*, 2011). Considering the functional trend of GPR3 and the cluster formation of CB_1 in oolemma followed by its disappearance at MI-stage, it can be assumed that both receptors internalization is a part of the mechanism controlling resumption of meiosis. In fact, it has been described in vertebrate oocytes the receptor endocytosis on which is dependent GVBD regulation (El-Jouni *et al.*, 2007; Nader *et al.*, 2014). Literature data demonstrate that in somatic cells the concomitant presence of G proteins and CB_1 in plasma membrane (Oddi *et al.*, 2012; 2018) and CB_2 in the cytoplasm (den Boon *et al.*, 2012) is essential for the physiological function of these CBRs. Thus, it can be hypothesized that also in oocytes occurs the functional co-localization of $G_{\alpha i}$-coupled receptors in the oolemma (CB_1) and in ooplasm (CB_2), suggesting that they could contribute to the fine regulation of oocyte cAMP levels, as shown by the present results. The aforementioned hypothesis is supported by the presence of microdomains useful for the production of cAMP localized both in the membrane and cytoplasm of fish and rat oocytes (Willoughby and Cooper, 2007; Bagavandoss and Grimshaw, 2012; Thomas, 2016). In addition, the results of SR1- and SR2-treated oocytes highlight the absence of any defect in PBI extrusion and in the structure of the meiotic spindle. Those data are in accordance with López-Cardona and coworkers, who found no defects in oocytes of

Cnr1- and *Cnr2*-KO mice, even if their depletion was highly effective in fertilization outcome and embryo quality during early development (López-Cardona *et al.*, 2017).

Completely novel is the role of GPR55 in meiotic spindle formation (MI and MII) through the experiments with ML193. Despite the unaltered percentages of PBI extrusion and chromosome alignment at metaphase plates, this antagonist caused a significant alteration (~75%) in spindle size of both MI- and MII-stage oocytes, compared to the much lower values of Ctr (MI, ~19%; MII, ~5%). This is the first time that the expression of GPR55 has been assessed in mammalian oocytes and it has been correlated with a correct organization of meiotic spindle. As a matter of fact, the interaction of a wide range of proteins regulates spindle length (Gaetz and Kapoor, 2004; Bennabi *et al.*, 2016; Chen *et al.*, 2018). Also, it is important to mention that one of the markers of oocyte quality is the normal spindle length (Tomari *et al.*, 2018). A recent finding points out to the importance of cytoplasmic composition in spindle sizing and progression timings, despite the nuclear components and cytoplasmic volume (Wang *et al.*, 2016). In accordance with this data, it is noteworthy to mention the pivotal role in microtubule length regulation covered by cytoplasmic proteins like dynein, dynactin, and NuMA, together with the γ-tubulin function in α-tubulin polymerization process (Radford *et al.*, 2017). Regarding GPR55 expression during the different stages of oocyte meiotic maturation, the results here presented highlight its ooplasm localization and its exponential increased expression throughout meiotic maturation until MII-stage. The higher levels in the stage before fertilization suggest that this receptor could have a role also in this process and in the early embryonic development. In fact, since, as GPR55 is coupled to G_q, its activation could control Ca^{2+} intracellular release from IP_3R-gated stores, as demonstrated for neurons (Lauckner *et al.*, 2008). Also, studies on human sperm reported the fine role of GPR55 in the regulation of Ca^{2+}-dependent processes, as sperm motility and capacitation (Schuel *et al.*, 2002; Amoako *et al.*, 2014). In this matter, it is important to mention that during fertilization one of the essential processes is the release of intracellular Ca^{2+} that stimulates cortical granules exocytosis to block polyspermy (Liu, 2011). The increased expression of GPR55 in MII oocytes could be connected to its role to induce Ca^{2+} intracellular release through G_q protein, even if this hypothesis has to be still investigated.

Although a limitation of this study is the use of a mouse model, the advantage relies on the possibility of testing experimental conditions that cannot be easily reproduced with human oocytes.

In conclusion, this thesis focuses on the characterization of the four main eCB-binding receptors' expression (CB_1, CB_2, GPR55, and TRPV1) throughout the different stages (GV, MI, and MII) of *in vivo* mouse oocyte meiotic maturation. The data here presented confirm the presence of CB_1 and CB_2 receptors and provide a description of their localization during the stages of *in vivo* maturation. Furthermore, in the current study, it has been demonstrated for the first time, GPR55 (gene and protein) expression and localization in immature (GV, MI) and mature (MII) oocytes. Molecular and immunofluorescence data exclude either the presence or modulation of the vanilloid receptor TRPV1 in any stage of *in vivo* maturation. By biochemical and morphological analyses, it has been evidenced that the three CBRs could play different roles in distinct processes essential for the oocyte meiotic maturation. Particularly, CB_1 and CB_2 are involved in the control of meiotic resumption (GVBD) through the maintenance of high levels of intra-cytoplasmic cAMP, while GPR55 function is centered on MI and MII spindle formation and organization.

Further studies are necessary to fully understand the importance of ECS in the process of oocyte meiotic maturation. It could be of interest to assess the effects of eCBs (e.g. AEA, 2-AG, Met-AEA) or of specific agonists of these receptors (e.g. THC, WIN 55,212-2, JWH-073, HU-308, LPI). Regarding CB1, it is of interest to understand the mechanism underlying its disappearance from oolemma during the GV-MI transition. About CB2 instead, it should be investigated the mechanisms through which this GPR can fulfill its role in controlling cAMP concentrations. Considering GPR55, it remains still unsolved the question of which molecular mechanisms are activated in the control of spindle formation during the MI- and MII-stage of oocyte meiotic maturation.

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